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Exploring Different Preservation Strategies
in a Pig Kidney DCD Autotransplant Model

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List of abbreviations

List of abbreviations

AOR: adjusted odds ratio

ADP: adenosine diphosphate

AMP: adenosine monophate

ATP: adenosine triphosphate

AUC: area under the curve

CA: cardiac arrest

CPR: cardiopulmonary resuscitation

DBD: donation after brain death

DCD: donation after circulatory death

DGF: delayed graft function

ECD: extended criteria donors

FMN: flavin mononucleotide

GST: glutathione-S-transferase

HIF-1 α : hypoxia inducible factor 1-alpha

HLA: human leukocyte antigen

HMGB1: High mobility group box 1

HMP: hypothermic machine perfusion

HO-1: Heme oxygenase 1

IRI: ischemia-reperfusion injury

NADH: reduced nicotinamide adenine dinucleotide

NAD⁺: oxidized nicotinamide adenine dinucleotide

NOX4: nicotinamide adenine phosphate oxidase 4

Nrf2: nuclear factor erythroid 2-related factor 2

List of abbreviations

NS: not significant

OR: odds ratio

pO₂: partial oxygen pressure

PNF: primary nonfunction

Prdx1: peroxiredoxin 1

RCT: Randomized controlled trial

ROS: reactive oxygen species

SCD: standard criteria donors

SCS: static cold storage

SOD1: superoxide dismutase 1

TLR-4: toll like receptor 4

WIT: warm ischemia time

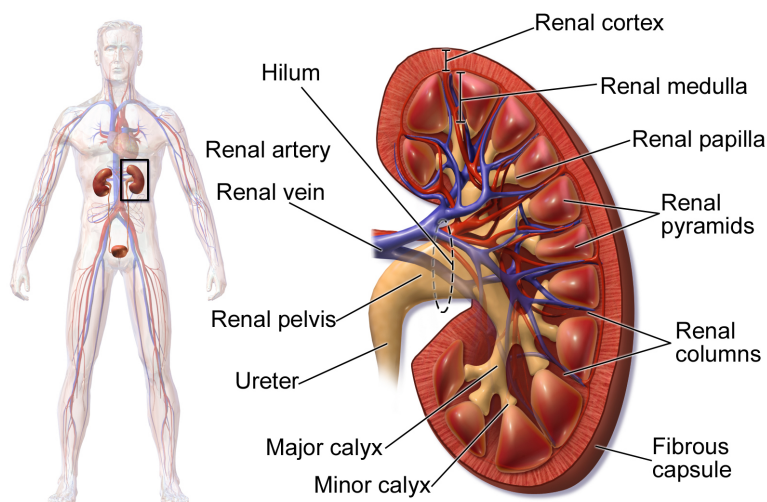
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Introduction

Chapter I: Background

1. Anatomy and function of the kidney

The kidneys are two bean-shaped organs. They are located on the right and the left in the retroperitoneal space. In adult humans they are about 11 centimeters in length (Figure 1). The kidneys receive blood from the renal arteries, left and right, which branch directly from the abdominal aorta. Despite their relatively small size, the kidneys receive approximately 20% of the cardiac output. Blood drains from the kidney, via paired renal veins into the inferior vena cava. Each kidney is attached to a ureter, a tube that carries excreted urine to the bladder. The kidney and nervous system communicate via the renal plexus, whose fibers course along the renal arteries to reach each kidney.



Kidney Anatomy

Figure 1: The kidneys lie in the retroperitoneal space behind the abdomen and act to filter blood to create urine.

The functional substance, or parenchyma, of the kidney is divided into two major structures: the outer renal cortex and the inner renal medulla. These structures take the shape of 8 to 18 cone-shaped renal lobes, each containing cortex surrounding a portion of medulla called a renal pyramid (Figure 2). Between the renal pyramids are projections of cortex called

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renal columns. Nephrons, the urine producing functional structures of the kidney, span the cortex and medulla.

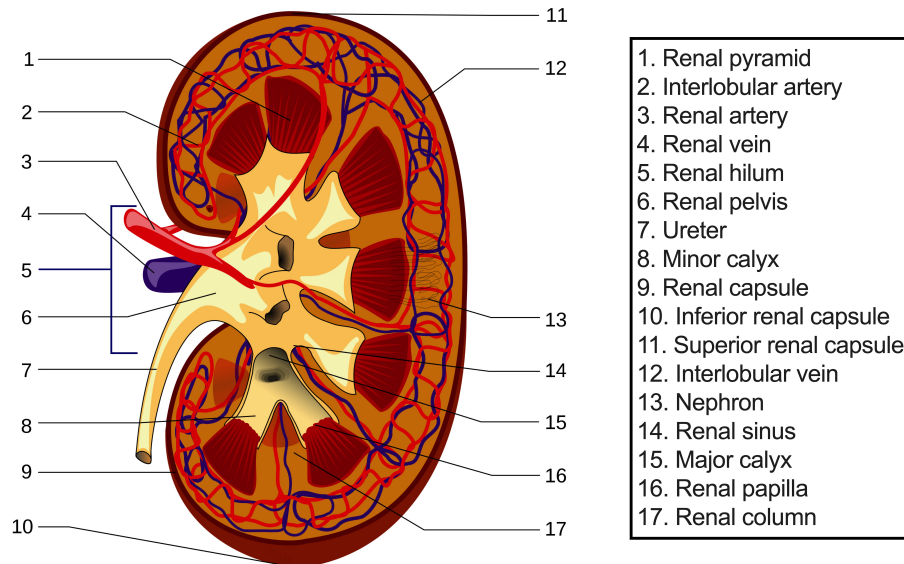


Figure 2: The gross anatomy of the kidney.

The kidneys excrete a variety of waste products produced by metabolism into the urine. The microscopic structural and functional unit of the kidney is the nephron (Figure 3). The nephron processes the blood supplied to it via filtration, resorption, secretion and excretion (Figure 4a). The consequence of those processes is the production of urine. Filtration takes place at the renal corpuscle and is the process by which cells and large proteins are retained while materials of smaller molecular weight are filtered from the blood to make an ultrafiltrate that eventually becomes urine. The kidney generates 180 liters of filtrate a day. Reabsorption is the transport of molecules from this ultrafiltrate into the peritubular capillary. Secretion is the reverse of reabsorption: molecules are transported from the peritubular capillary through the interstitial fluid then through the renal tubular cell into the ultrafiltrate. Excretion is the last step: the ultrafiltrate passes out on the nephron and travels through a tube called the collecting duct, which is part of the collecting duct system, and then to the ureters where it is named urine. Figure 4b provides a more detailed overview of secretion and reabsorption of various substances throughout the nephron.

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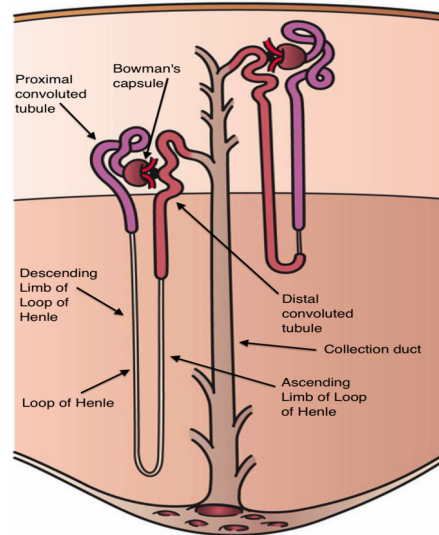


Figure 3: The nephron is the functional unit of the kidneys.

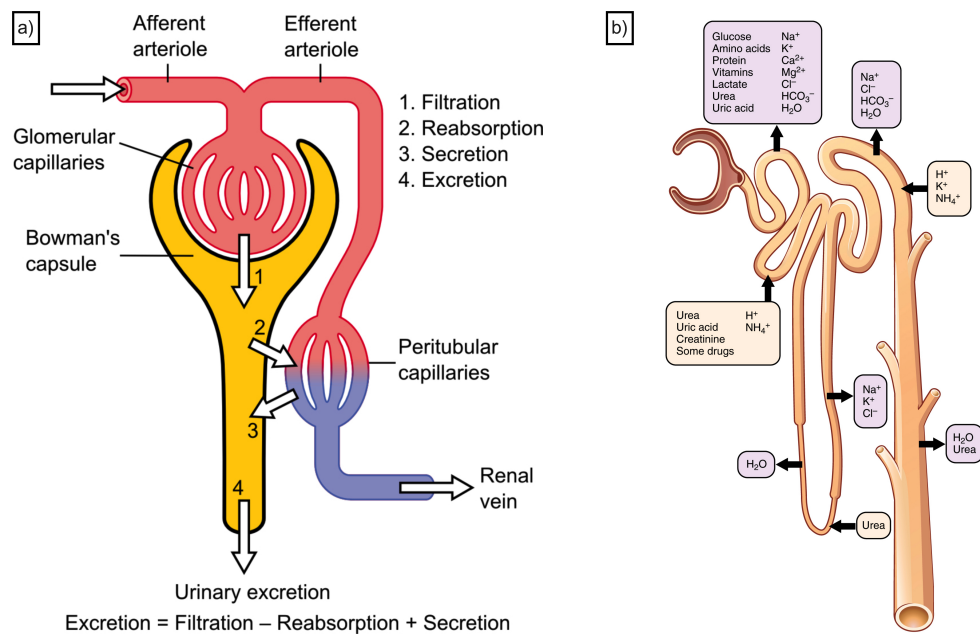


Figure 4: Four main processes are involved in the creation of urine (a) and a detailed overview of secretion and reabsorption of the principal substances through the nephron (b).

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The kidney is involved in maintenance of whole body homeostasis and acid-base homeostasis (reabsorb and regenerate bicarbonate from urine, and excrete hydrogen ions and fixed acids into urine), hormone secretion (erythropoietin, calcitriol and renin), blood pressure regulation (renin-angiotensin system) and regulation of osmolality.

2. Indications for kidney transplantation

Chronic kidney disease can be classified as illustrated in Table 1.¹

GFR category	Terms	Glomerular filtration rate (ml/min/1.73m ²)
G1	Normal or high	≥90
G2	Mildly decreased	60-90
G3a	Mildly to moderately decreased	45-59
G3b	Moderately to severely decreased	30-44
G4	Severely decreased	15-29
G5	Kidney failure	<15

Table 1: Staging of chronic kidney disease.

G, Grade; GFR, glomerular filtration rate.

The indication for isolated kidney transplantation is end-stage renal disease (kidney failure), regardless the primary cause. This is defined as a glomerular filtration rate below 15 ml/min/1.73 m². The most common known causes of renal failure are diabetes and high blood pressure. Other causes are infections, autoimmune conditions such as chronic glomerulonephritis and lupus. Genetic causes include polycystic kidney disease, and a number of inborn errors of metabolism. The most commonest cause is idiopathic (i.e. unknown).

A succesful kidney transplant results in improved patient survival (when compared with maintenance dialysis), quality of life and cost reduction compared to chronic dialysis.²⁻¹²

3. History of kidney transplantation

The era of modern kidney transplantation started in 1950s after decades of experimental work. In particular, the development of surgical techniques for vascular anastomosis by Alexis Carrel (1873-1944)¹³ lead to the first allotransplantation in the world of a kidney removed from a voluntary living

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donor in 1952 at the Necker hospital in Paris by Jean Hamburger, although the kidney failed after 3 weeks.¹⁴ The first truly successfully transplanted kidney between identical twins was performed by Joseph Murray, John Merrill and their team on December 23, 1954.¹⁵ Both, Alexis Carrel and Joseph Murray received afterwards the Nobel price for their pioneering work. From then on, many such transplantations were performed successfully in Boston.¹⁶ In that time, no attempt was made to cool or perfuse the kidney between procurement and transplantation of the organ and from the 1950 until early 1960s the majority of performed allotransplantations failed because of rejection in the absence of immunosuppressive drugs.¹⁷

Irradiation in combination with allograft bone marrow infusion used for immunosuppression was carried out in Paris, Boston and elsewhere in the late 1950s.¹⁷ This regimen was too difficult to control, and graft-versus-host disease was inevitable. However, this approach was used until 1962, the year of the first general availability of azathioprim (Imuran®) after preclinical work of Roy Calne in dogs.¹⁸⁻²⁰ This led to the first extended successes with human kidney allografts.²¹ Shortly afterwards, it was discovered that steroids, notably prednisolone, when given with azathioprine had a powerful synergistic effect. The regular use of both drugs together became a standard regime after reports by Starzl²² and Goodwin²³, until azathioprine was displaced by cyclosporine much later in 1978.²⁴ And since the introduction of tacrolimus in 1997, tacrolimus-based immunosuppressive regimens became the cornerstone to prevent graft rejection until today in two-third of the transplant centers worldwide.

With the growing success of clinical transplant programs worldwide due to immunological and surgical advancements, the next issue was the preservation of kidney grafts. In the last 3 decades, the research focus was to improve preservation solutions to improve static cold storage preservation. However due to the deteriorating quality of available deceased donor organs a revival of machine perfusion preservation strategies was seen from the beginning of the twenty-first century and is still ongoing.

4. Donor shortage and type of donors

During the last decade, the gap between organ demand and organ availability was increasing enormously, as illustrated in Figure 5. The high demand of organs is partly attributed to the aging population with increased incidence of diseases that lead to end-stage organ failure, such as diabetes. On the other hand, in the past decades a relative reduction in

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numbers of organ donors has been observed due to the improvements in neurosurgical treatment, intensive care management and road safety.

The organ shortage is predicted to worsen over the course of the next decades. Therefore, one of the greatest challenges of the transplant community will be the search for alternative organ sources and the more optimal preservation of these organs.

Donor types can be divided into living donors and deceased donors. Living donation provides excellent short- and long-term results in the recipient.²⁵ The incidence of living donor kidney transplantation in the Eurotransplant region is increasing slowly over the last decades, but unfortunately the impact on the waiting list is relatively minimal (see also Figure 5). Despite improvements in outcome and increased use of non-biologic (=altruistic) donors, the number of living donation transplants in the United States has declined over the last decade. This decline was greater in children than adults.²⁵

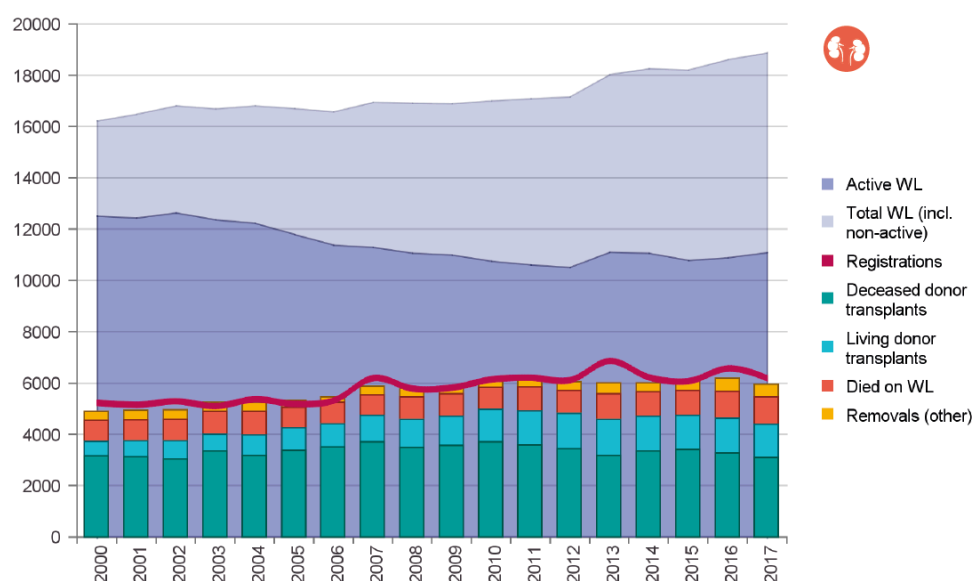


Figure 5. Dynamics of the Eurotransplant kidney transplant waiting list and transplants between 2000 and 2017 (Eurotransplant Annual Report 2017).

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Deceased donors can be divided into 2 types: donation after brain death (DBD) and donors after circulatory death (DCD). Brain death donors are donors who meet the neurological criteria of brain death.²⁶ Brain death donors with standard criteria (e.g. young trauma victim, less than 50 years old) are nowadays less frequent due to the decrease of traffic accidents, improved intensive care management and evolution in neurosurgical techniques. Extended criteria donors (ECD)(defined as a donor age ≥ 60 years or donor age ≥ 50 years plus 2 of the 3 following features: history of arterial hypertension, a raised terminal serum creatinine (>1.5 mg/dl) and death from a cerebrovascular accident) are therefore more frequently used compared to standard criteria donors (SCD) in brain death donors.²⁷ However, organs transplanted from ECDs have poorer short- and long-term outcomes compared to organs obtained from "optimal" brain death donors or living donors.^{2,28} In contrast to the classical brain death donors, the donor pool was expanded since 1995 by the use of donors who have been declared dead based on cardiocirculatory rather than neurological criteria. These are mostly donors with irreversible brain injury who do not meet the criteria for brain death and who experience cardiocirculatory arrest after withdrawal of ventilator support. Donors after circulatory death are classified according to the Maastricht classification.²⁹ This classification was modified during the 6th International Conference on Organ Donation after Circulatory Death in Paris 2013 as illustrated in Table 2.³⁰ Legislation in some countries allows euthanasia and subsequent organ donation included in the table as the fifth category.^{30,31}

Category I <i>uncontrolled</i>	Found dead IA. Out-of-hospital IB. In-hospital	Sudden unexpected CA without any attempt of resuscitation by a life-medical team
Category II <i>uncontrolled</i>	Witnessed cardiac arrest IIA. Out-of-hospital IIB. In-hospital	Sudden unexpected irreversible CA with unsuccessful resuscitation life-by a life-medical team
Category III <i>Controlled</i>	Withdrawal of lifesustaining therapy	Planned withdrawal of life-sustaining therapy; expected CA
Category IV <i>Controlled</i>	Cardiac arrest while life-brain dead	Sudden CA after brain death diagnosis during donor life-management but prior to planes organ recovery
Category V <i>Controlled</i>	Includes patients who grant access to medically assisted circulatory death	

Table 2: The modified Maastricht classification of DCD (table from³⁰). CA, cardiac arrest.

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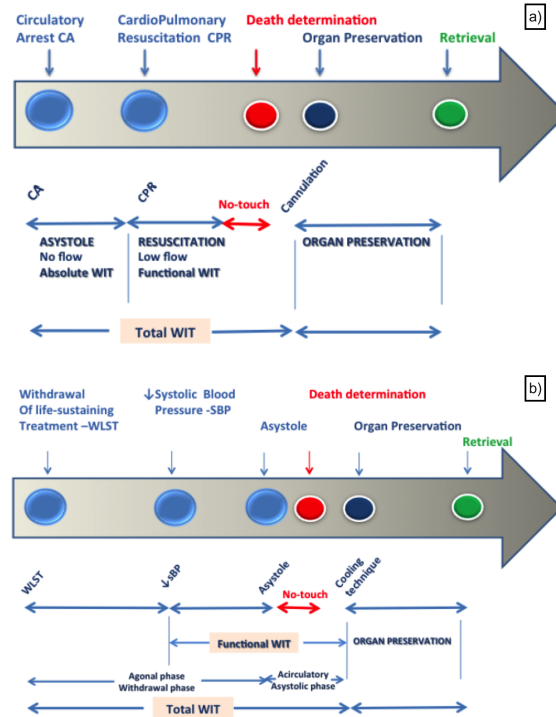


Figure 6. The difference in warm ischemia time in uncontrolled (a) and controlled (b) donation after circulatory death (figure from³⁰). CA, circulatory arrest; CPR, cardiopulmonary resuscitation; WIT, warm ischemia time.

One of the most important differences between DBD and DCD donation is the warm ischemia which exacerbates the ischemia-reperfusion injury in DCD donation and is responsible for the higher incidence of delayed graft function (DGF). No warm ischemia exists before the start of in situ organ cooling in DBD before procurement because organs are well perfused and oxygenated by the beating heart until in situ flush with cold preservation solution during procurement. Warm ischemia time is much more important in DCD donation. The difference in warm ischemia time between uncontrolled and controlled DCD is illustrated in Figure 6. The no-touch period is, depending on the jurisdiction in a range from 2 min (e.g. USA; 3-5 in Belgium) to 20 min (e.g. Italy). Functional warm ischemia time in controlled DCD donors starts when systolic blood pressure is less than 50-60 mmHg.³⁰

The use of these more marginal donors (ECDs and DCDs), especially this extra warm ischemia time in DCDs exacerbates the problems of

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preservation and ischemia-reperfusion injury (IRI), because preservation takes place on an existing background of cellular injury and energy depletion. Therefore, short- and long-term results are poorer using these higher risk donors.^{28,32-36} Current research, including the topic of this thesis, is focusing on improving preservation of these higher risk organs to *decrease delayed graft function* (see Introduction, Chapter II) and *ischemia-reperfusion injury* (see Introduction, Chapter III) with the aim to improve early and late function of the transplanted kidney.

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Chapter II: Delayed graft function

Early graft dysfunction is often classified into immediate, slow, delayed, or in most severe cases, primary nonfunction (PNF)(Figure 7).³⁷

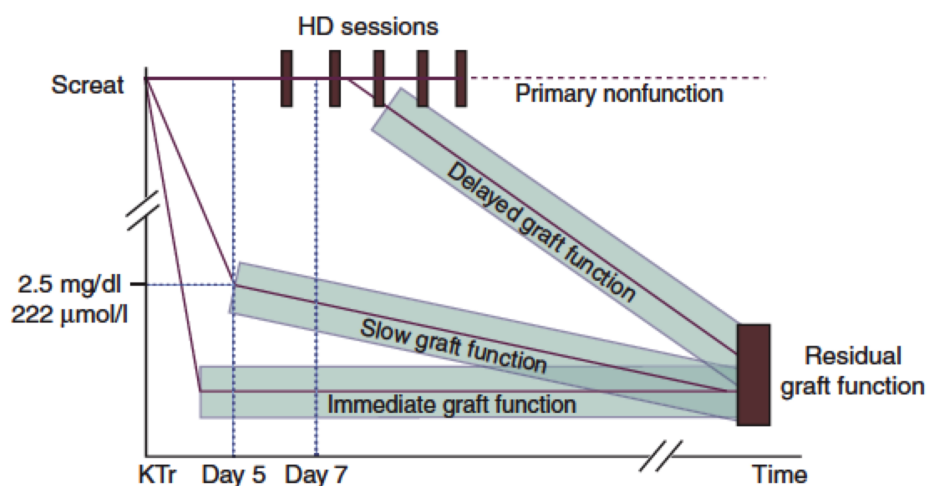


Figure 7: Schematic representation of renal function recovery after kidney transplantation. Recovery may be immediate, slow (serum creatinine > 2.5 mg/dl within 5 days), or delayed (most often defined by at least one dialysis session within 7 days), or never occurring (primary nonfunction)(figure from³⁷). HD, hemodialysis; KTr, kidney transplantation; Screat, serum creatinine.

Due to its complex pathophysiology, no uniform definition of early graft dysfunction exists, which explains why currently more than 18 definitions coexist.³⁸ The most frequent definition is based on dialysis requirements after transplantation (at least one dialysis session during the first 7 days after transplantation).³⁸ For the same reason, the incidence of DGF is also quite variable exceeding 20% for brain death donors³⁹ and 50-90% for DCD kidney transplants.^{38,40,41}

Table 3 shows the risk factors for the development of DGF.^{42,43} The main donor factors increasing the risk of DGF are increasing donor age, donor type, and quality of pre-kidney procurement care.^{37,44} The risk of DGF increases from living donor kidneys to deceased donors (SCD<ECD^{28,45}<DCD<ECD/DCD).^{40,46,47} Modifiable preservation risk factors for DGF are warm ischemia time, cold ischemia time^{39,48-50}, cold storage solution^{51,52} and machine perfusion versus static cold storage.^{37,42} Important recipient factors influencing the risk of DGF include male gender, body

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mass index >30, African-American ethnicity, history of diabetes, anti-human leukocyte antigen immunization, and requirements for dialysis before transplantation.⁵³

Donor related	Recipient related
Female gender	Male gender
Increasing age	Black race
Body mass index	Body mass index
Deceased versus living donation	Previous transplant
Donation after cardiac death	Diabetes
Increasing donor serum creatinine	Pre-transplant dialysis versus none
Cause of death (anoxia vs cerebrovascular)	Duration and type of dialysis
Duration of intensive care stay	Residual diuresis
Duration of brain death	Pre-transplant transfusion
Diabetes	Pre-transplant blood pressure
History of hypertension	Pre-transplant lipid profile
Graft atherosclerosis	Cardiac function
Preservation	Transplant-related
Cold ischemia time	Sensitization
Warm ischemia time	Human Leukocyte Antigen (HLA) mismatch
Preservation solution	ABO blood group incompatible
Machine perfusion vs static cold storage	

Table 3: Risk factors for the development of delayed graft function (table created from^{42,43}).

Once hyperacute rejection, vascular and urinary tract complications, and hyperkalemia are excluded as cause of early graft dysfunction or indication for dialysis, DGF is primarily a consequence of ischemia-reperfusion injury (see also Introduction, Chapter III) resulting in *post-ischemic acute tubular necrosis*.

The *short-term clinical consequences of DGF* are the need for several post-transplant dialysis sessions leading to increased morbidity, increased length of hospitalization, and hence increased cost.^{39,46,48,54,55} One of the major concerns regarding the increasing use of kidneys from DCD donors is the high incidence of DGF observed in such transplants. Determination of the impact of DGF on allograft survival is complicated by the fact that DGF

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is a clinical entity caused by a complex series of factors and events that individually may or may not have a durable impact on long-term outcome. Epidemiological studies have shown an independent association between DGF and acute rejection in DBD donors, presumably from a reduction in immunosuppression during periods of DGF.⁵⁶⁻⁵⁸

However, the *long-term impact of DGF* is more controversial. The detrimental impact of DGF on graft outcome of kidneys from DBD donors is well documented, with one large meta-analysis reporting an increase in relative risk of graft loss by up to 41% at 3 years of follow up.³⁸ However, using kidneys originating from DCD donors, the adverse impact of DGF is probably not the same. Summers et al demonstrated in a UK cohort a comparable 5-years estimated glomerular filtration rate between DCD (n=3626) and DBD (n=9684) donors (49.6 versus 48.1 ml/min/1.73m², respectively)(p=0.06), despite a significantly higher incidence of DGF in the DCD group (48.5% versus 24.9%).⁵⁹ Gagandeep et al demonstrated similar results analyzing the US national database.⁶⁰ These results were in line with other studies.⁶¹⁻⁶⁵ This suggest a different impact of DGF on kidneys originated from DCD donors compared to DBD donors.

An important related question is whether the increased short-term morbidity and mortality associated with DGF negates the survival advantage of transplantation over maintenance dialysis, especially in high-risk donors. Gill et al found that the DGF associated risk of graft failure was the greatest in the first posttransplant year, and in patients with concomitant rejection (hazard ratio: 8.22, 95% confidence interval: 4.76-14.21).⁶⁶ In contrast, the DGF-associated risk of graft failure after the first posttransplant year in patients without acute rejection was far lower (hazard ratio: 1.15, 95% confidence interval: 1.02-1.29). Recipients of transplants complicated by DGF still derived a survival benefit when compared with patients who received treatment with dialysis irrespective of donor quality as measured by the Kidney Donor Profile Index.⁶⁶ This justifies the acceptance of kidneys at high risk of DGF.

Chapter III: Ischemia-reperfusion injury

Cellular damage after reperfusion of ischemic tissue is defined as ischemia-reperfusion injury (IRI).⁶⁷ Although restoration of blood flow to an ischemic organ is essential to prevent irreversible cellular injury, reperfusion may augment tissue injury in excess of that produced by ischemia alone. Renal IRI is until today unavoidable during the process of organ procurement and transplantation and is one the most important risk factors for DGF, acute kidney injury⁶⁸, acute and chronic rejection^{69,70} and long term renal graft survival.⁷¹

A brief summary of the pathophysiology of IRI is illustrated in Figure 8.⁷²

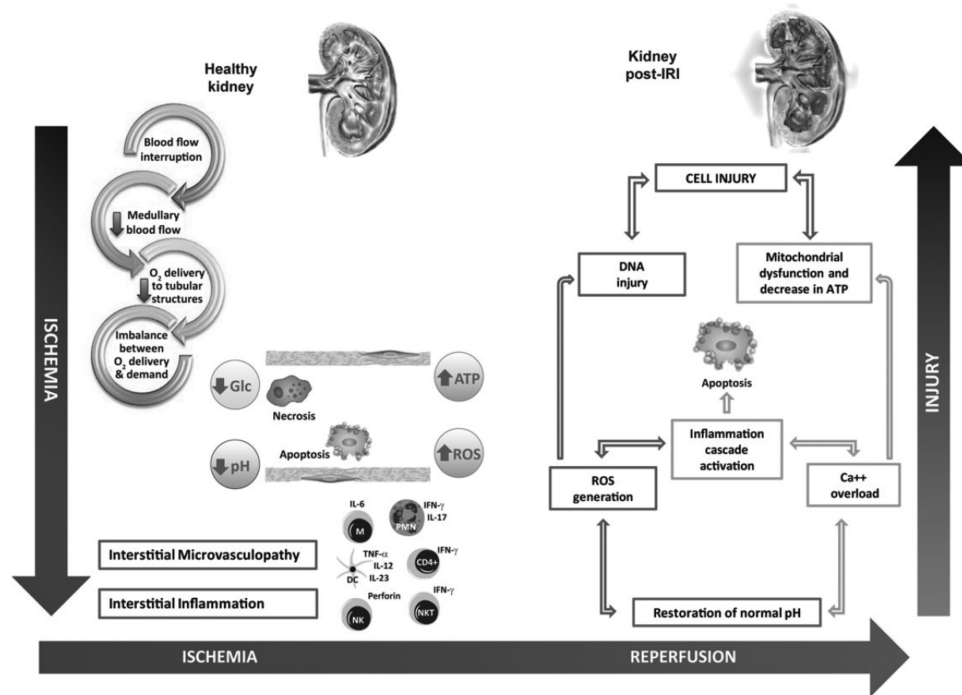


Figure 8: Overview of pathophysiology of ischemia-reperfusion injury. The blood flow is interrupted and causes an imbalance between O₂ delivery and demand. Hypoxia, glucose depletion, acidosis and reactive oxygen species production contribute to cell death. Tubular epithelial and vascular endothelial cells release pro-inflammatory cytokines (interstitial inflammation) that are responsible for endothelial cell swelling with a consequence prolonged ischemia and slower reperfusion (interstitial microvasculopathy). During reperfusion, the restoration of pH induce Ca²⁺

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delivery by depleted cells, producing Ca^{2+} overload and increased oxygen-derived free radical formation that both contribute to an inflammatory cascade and cell injury (figure from⁷²). ATP, adenosine triphosphate; Ca^{++} , calcium ions; DNA, deoxyribonucleic acid; Glc, glucose; ROS, reactive oxygen species; IRI, ischemia-reperfusion injury; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.

Today, the gold standard for deceased donors is to procure the kidneys after in situ cold flush of the organs with a preservation solution in combination with topical cooling. The aim is to reduce metabolism during cold preservation at 4°C based on the principle that metabolism is slowed by almost 2-fold for each 10°C drop in temperature (static preservation method) to keep metabolic rate at about 10% during cold preservation.⁷³ According to the definition, IRI starts with a *period of ischemia*. During procurement of the kidney, the blood flow is interrupted and causes an imbalance between O_2 delivery and demand. The consequent hypoxia, glucose depletion, acidosis and reactive oxygen species (ROS) production contribute to cell death. In addition, tubular epithelial and vascular endothelial cells release pro-inflammatory cytokines (interstitial inflammation) that are responsible for endothelial cell swelling.⁷⁴ The kidney will subsequently be stored on static cold storage and/or machine perfusion conditions before transplantation (see Introduction, Chapter IV). The second phase of the IRI is the *reperfusion period* after reimplantation of the kidney in the recipient. The restoration of pH induce Calcium delivery by depleted cells, producing Ca^{2+} overload and increased oxygen-derived free radical formation that both contribute to an inflammatory cascade and cell injury.

It is now clear that much of the ischemic damage is mediated by mitochondrial oxidative stress and reactive oxygen species during reperfusion.⁷⁴⁻⁷⁶ This topic will be discussed extensively in the discussion sections of the first 3 studies of the thesis.

Multiple factors influence the degree of IRI after transplantation and subsequent graft functions as illustrated in Figure 9.

IRI attacks in particular endothelial cells and tubular epithelial cells.^{37,77} Tubular cell death by necrosis and apoptosis is a central feature of renal IRI. Recent research has challenged traditional views of cell death by identifying new pathways in which cells die in a regulated manner but with the morphological features of necrosis.⁷⁸ This regulated necrosis takes several forms, with necroptosis and ferroptosis being the best described.⁷⁸ However, IRI is a complex process involving also the innate (e.g. Toll-like

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receptors, dendritic cells, inflammasomes and the complement system) as well as the adaptive immune system (e.g. CD4 and CD8 T-cells).^{37,77} The innate immune system should not be seen as a separate system, but rather as an overlapping response to disturbed tissue integrity.

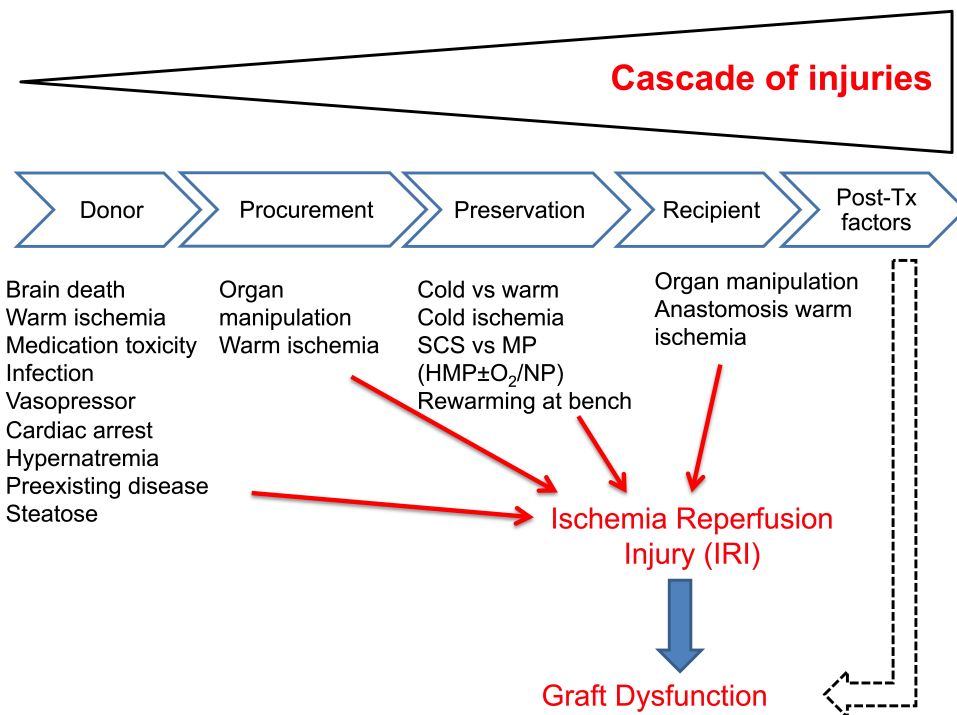


Figure 9: Factors influencing the degree of ischemia-reperfusion injury after transplantation and subsequent graft function (figure adapted from Pirenne J, presented at the 17th Congress of the European Society for Organ Transplantation 2015). HMP, hypothermic machine perfusion, NP, normothermic perfusion; SCS, static cold storage; Tx, Transplant.

The reason why the kidney is so susceptible to ischemic damage seems paradoxical in an organ which blood flow far exceeds its metabolic needs. It has been proposed that unlike the well-perfused renal cortex, the renal medulla normally operates just above the threshold for hypoxia and that further reductions in renal perfusion cause hypoxic injury in this metabolically active region.⁷⁹ An interesting alternative proposal is that the true cause of ischemic injury is not a simple mismatch between medullary

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metabolic demand and oxygen supply, but rather the susceptibility of the outer medulla to vascular congestion. The capillary plexus of the renal outer medullary region is especially prone to vascular congestion during periods of ischemia. It is the failure to restore the circulation to the outer medulla that mediates complete and prolonged ischemia to much of this region, leading to injury and tubular cell death.⁷⁹

The severity of acute kidney injury due to IRI can influence the graft outcome. However, the kidney has the ability to repair itself in case of mild acute kidney injury with no or mild clinical DGF and no consequences on long term graft outcome. When the injury is more severe or in the presence of kidney abnormalities, the repair process can lead to fibrosis, which can facilitate progression to chronic allograft dysfunction.^{77,80-82} Epithelial-to-mesenchymal transition of injured epithelial cells plays a major role in the development of interstitial fibrosis. In this process, tubular epithelial cells progressively lose their epithelial characteristics and acquire features of mesenchymal cells.^{77,82,83} Hypoxia and several signal pathways are involved in this process.^{77,83}

Early IRI, if the kidney is not able to full repair does contribute to later graft loss through reduction of renal functional mass, graft vascular injury, and chronic hypoxia, as well as subsequent fibrosis. IRI is also known to induce kidney allograft dysfunction and acute rejection reducing graft survival.⁷¹

Due to its multifactorial underlying mechanism and complexity, IRI provides different potential targets to prevent or ameliorate renal IRI before, during and after transplantation, currently in preclinical and clinical research, as illustrated in Table 4.⁸⁴

Exploring different machine perfusion modalities to improve IRI and subsequent initial graft function will be the main subject through the different studies of this thesis.

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Renal ischemia-reperfusion injury		
Before Transplantation	During preservation	After transplantation
Donor management	Preservation/storage	Mesenchymal stem cell therapy
Dietary preconditioning	Machine perfusion	Pharmacological treatment
	Ischemic pre- / post conditioning	microRNA's
	Mesenchymal stem cell therapy	
	Pharmacological treatment	
	micro RNA's	

Table 4: Overview of various therapies before, during and after kidney transplantation currently explored to ameliorate renal ischemia-reperfusion injury in preclinical and clinical research (adapted from⁸⁴).

Chapter IV: Evolution of kidney preservation techniques

1. Static cold storage

Static cold storage (SCS) on ice is still worldwide the 'gold standard' to preserve kidneys for transplantation. This technique is simple and easy (i.e. during procurement and transport of the organ) and, because of its low cost, it's still frequently used worldwide. The drive to use hypothermia (4°C) for organ preservation is based on the fact that lowering the temperature slows down cell metabolism. This is based on 2 relations: 1) the van't Hoff equation: at 4°C a chemical reaction will only be 40% as effective as the same reaction taking place at 37°C, and 2) the Arrhenius relation, quantifying the impact of temperature on the speed of the chemical reaction, highlighting that a reaction taking place at 4°C is 90% slower than the same reaction at 37°C.⁸⁵ The main disadvantage of this static preservation technique is that hypothermia worsens ischemic injuries through: 1) reduction of ATP synthesis and metabolic activity, 2) reduced Na⁺-K-ATPase activity, which induces osmotic perturbation, 3) mitochondrial perturbations, 4) decreased cell survival and 5) endothelial activation. With the increasing acceptance of high risk kidneys, another major disadvantage of static cold storage is the lack of viability assessment during and after preservation to evaluate "transplantability".

2. Hypothermic machine perfusion

The concept of hypothermic machine perfusion (HMP) is not a new one. Early research into HMP dates back to the 1960s.^{86,87} Folker O Belzer designed the first clinical machine perfusion device used during the first successful human transplantation of a 17-hour hypothermically machine-perfused kidney in 1968.⁸⁸ This machine perfusion set-up was transformed to a smaller portable device and used for the first time to transport a kidney over the Atlantic Ocean, from New York to Leiden in 1971. This kidney was successfully transplanted after 37 hours of hypothermic preservation. Hypothermic machine perfusion was used in the 1970s in the United States and Europe but because successful long term graft outcome after static cold storage⁸⁹ in combination with its more easier logistics and lower costs, machine perfusion disappeared in clinical transplantation for the next 2 decades.

High risk donors (ECD, DCD, ECD/DCD) are currently more and more accepted to compensate organ shortage. Therefore, the transplant community is *exploring different modalities of machine perfusion to improve organ preservation and ex vivo graft assessment to decrease IRI* with the

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aim to increase acceptance rate of currently discarded organs and improve early and late graft function. As mentioned before, compared with DBD kidneys, DCD kidney grafts have a significantly higher incidence of DGF and PNF (13-35% and 1-10% versus 28-88% and 1-18%, respectively).^{40,90} Currently available hypothermic kidney machine perfusion devices are shown in Figure 10.

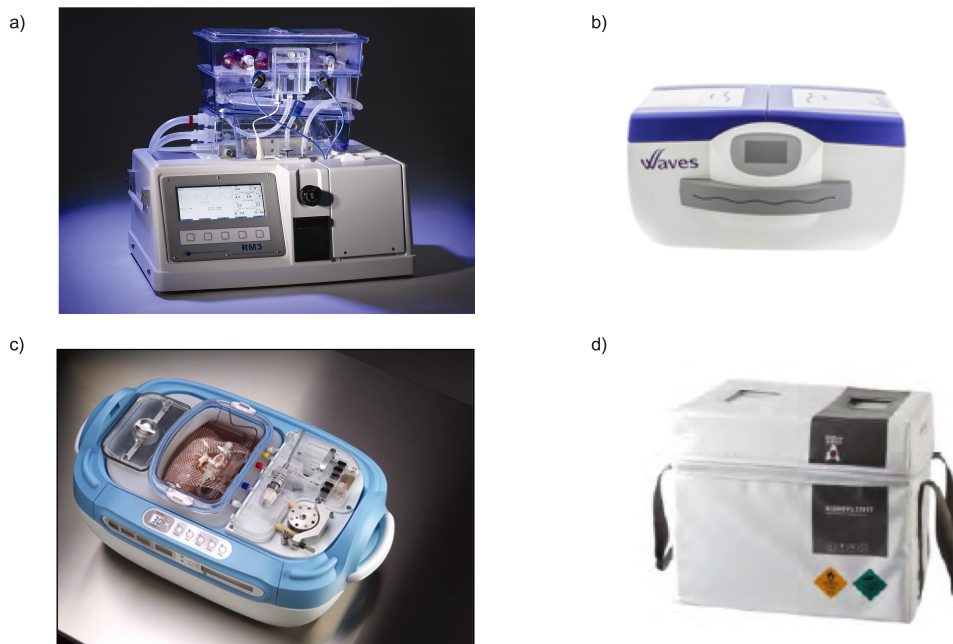


Figure 10: Currently available hypothermic machine perfusion devices for clinical kidney preservation: a) RM3® Kidney Perfusion System (Waters Medical systems, Birmingham, AL, USA), b) WAVES® transportable Kidney Perfusion (Waters Medical systems, Birmingham, AL, USA), c) LifePort Kidney Transporter® (Organ Recovery Systems, Itasca, IL, USA), d) Kidney Assist® (Organ Assist, Groningen, the Netherlands).

Operating mechanism of HMP

The exact working mechanism of HMP is probably multifactorial and based on mechanical vasodilation and molecular vasoprotection (decreased vimentin, fibrosis, endothelin, innate immunity, Toll like receptor 4 (TLR-4), High Mobility Group Box 1 (HMGB1) and increased protective endothelial

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genes, Hypoxia-Inducible Factor 1-alpha (HIF-1 α) and nitric oxide signaling).⁹¹⁻⁹⁷

Physical parameters to predict graft function

In the United states, more than 15% of the machine-perfused kidneys are discarded annually, partly based on elevated renal resistance.⁹⁸ However, the only prospective study to determine the true value of the renal resistance during HMP is the Eurotransplant machine perfusion trial.⁹⁹ In this trial, the preservation method was not revealed at the time of the organ offer and in case of HMP, the kidney was accepted only based on donor data because renal resistance was unknown to the clinician. Renal resistance at the end of HMP was an independent risk factor for the development of DGF (Adjusted odds ratio (AOR) 38.1 (1.56-93.4)(p=0.03)). However, the predictive power of renal resistance was low (Area under the curve (AUC) of the receiver operating characteristic curve (ROC) 0.58). This means that renal resistance has a limited value to predict DGF and cannot be used on its own to predict graft viability and accept or discard kidneys on HMP.⁹⁹

Value of perfusate biomarkers.

A more valuable approach is offered by the machine giving access to the organ perfusate throughout the preservation period, allowing the measurements of biomarkers predictive of transplantation outcome. Perfusate analysis of the above mentioned Eurotransplant machine perfusion trial demonstrated that glutathione-S-transferase (GST), N-acetyl- β -D-glucosaminidase (NAG) and heart-fatty acid binding protein (H-FABP) were independent predictors for DGF but not for PNF and graft survival.¹⁰⁰ Lactate dehydrogenase, aspartate aminotransferase, and alanine-aminopeptidase had no independent prognostic value. Rapid metabolomic analysis in the perfusate by nuclear magnetic resonance demonstrated in a preclinical model, that the levels of several metabolites during HMP could be an interesting tool to access graft quality and functional recovery.¹⁰¹

Clinical evidence

Although HMP technology has been utilized for more than 20 years, only a few studies compared HMP versus static cold storage in a well designed randomized controlled trial (RCT). An overview of the most relevant RCTs, large registry data and meta-analysis over the last decade is provided in Table 5, 6 and 7, respectively (only risk for DGF is shown). We can conclude that HMP is superior to static cold storage in deceased donor kidney transplantation to decrease DGF. This is true for DBD and DCD

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donors. However graft survival benefit could not be demonstrated in most RCTs, also large registry data were not able to demonstrate graft survival benefit in favor of HMP (data not shown in Table 5).

Table 5: Randomised clinical trials in kidney transplantation: HMP versus SCS.

Author, year	Donor type	SCS group (n)	HMP group (n)	Risk for DGF: HMP vs SCS	1y death censored graft survival, HMP vs SCS
Moers, 2009 ¹⁰²	All*	336	336	AOR 0.57 (0.36-0.88)(p=0.01)	94% vs 90% (p=0.04)
Gallinat, 2012 ¹⁰³	DBD/DCD	85	85	OR 0.68 (0.32-1.43)(p=0.32)	89% vs 81% (p>0.05)
Tedesco-Silva, 2017 ¹⁰⁴	DBD	80	80	OR 0.49 (0.26-0.95)(p=0.03)	NA (p=0.241)
Jochmans, 2010 ¹⁰⁵	DCD	82	82	AOR 0.43 (0.20-.89) (p=0.025)	94% vs 95% (p=NS)
Watson, 2010 ¹⁰⁶	DCD	45	45	OR 1.14 (0.38-3.49)(p=NS)	93% vs 98% (p=0.03)

Tabel 5: Risk for DGF using hypothermic machine perfusion versus static cold storage in the most relevant RCTs from 2009-2019 and influence on graft survival. AOR, adjusted odds ratio; DBD, donation after brain death; DCD, donation after circulatory dead; DGF, delayed graft function; ECD, extended criteria donor; NA, not available; NS, not significant; OR, odds ratio; SCS, static cold storage.

* included DBD/ECD/DCD

The difference of impact of machine perfusion between the Eurotransplant machine perfusion, 2009¹⁰² and the UK trial, 2010 is probably related to the different study design. In the Eurotransplant machine perfusion trial kidneys were pumped during the entire period of preservation. In contrast in the UK trial, the kidneys were only pumped in the recipient center after a period of static cold storage for transport. However, other factors like longer warm and cold ischemia times in the Eurotransplant trial compared with the UK trial could also influence this difference in DGF rate between both studies.

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Table 6: Large registry databases in kidney transplantation: HMP versus SCS.

Author, year	Donor type	SCS group (n)	HMP group (n)	Risk for DGF: HMP vs SCS
Cannon, 2013 ¹⁰⁷	All*	32351 (62.1%)	19372 (37.9%)	OR 0.63 (0.60-0.66)(p<0.0001)
Gill, 2014 ¹⁰⁸	All*	SCD 59060 ECD 9318 DCD 2532	SCS 12132 ECD 5804 DCD 586	SCS: OR 0.69 (0.64-0.75) ECD: OR 0.59 (0.53-0.66) DCD: OR 0.80 (0.69-0.93)
Cannon, 2015 ¹⁰⁹	SCD DBD	8929	8929	OR: 0.59 (<0.001)
Savoye, 2019	ECD	3515	801	OR 0.49 (0.40-0.60) (p<0.001)

Table 6: Risk for delayed graft function using hypothermic machine perfusion versus static cold storage in large registry databases during the last decade. DBD, donation after brain death; DCD, donation after circulatory death; DGF, delayed graft function; ECD, extended criteria donor; OR, odds ratio; SCD, standard criteria donors; SCS, static cold storage.

* included DBD/ECD/DCD

Table 7: Metaanalysis in kidney transplantation: HMP versus SCS.

Author, year	Donor type	Variable	Risk ratio (Confidence interval)
Lam, 2013 ¹¹⁰	All*	DGF PNF 1y graft loss	0.83 (0.72-0.96)(p=0.01) 0.76 (0.45-1.28)(p=0.30) 0.87 (0.64-1.19)(p=0.39)
O'Callaghan, 2013 ⁵¹	All*	DGF PNF	0.81 (0.71-0.98)(p=0.002) 1.15 (0.46-2.90)(0.767)
Jiao, 2013 ¹¹¹	ECD	DGF PNF 1y graft survival	0.59 (0.54-0.66)(p<0.001) 0.54 (0.21-1.40) (p=0.40) 1.12 (1.03-1.21)(p=0.005)
Deng, 2013 ¹¹²	DCD	DGF PNF 1y graft survival	OR 0.56 (0.36-0.86) (p=0.008) OR 1.30 (0.49-3.44)(p=0.60) OR 0.64 (0.28-1.46)(p=0.29)

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Bathini, 2013 ¹¹³	DCD	DGF 1y graft survival	OR 0.64 (0.43-0.95)(p=0.03) OR 0.74 (0.48-1.13)(p=0.17)
Martinez, 2018 ¹¹⁴	All*	DGF PNF	All 0.79 (0.71-0.88) DBD 0.85 (0.74-0.98) DCD 0.75 (0.61-0.92) 0.92 (0.73-1.16)(p=NS)
Bellini, 2019 ¹¹⁵	All*	DGF PNF Acute rejection 1y graft survival	OR 0.57 (0.45-0.72)(p=<0.0001) OR 0.035 (1.02-2.53)(p=0.003) OR 0.91 (0.66-1.27)(p=NS) OR 1.61 (1.02-2.53)(p=0.04)
Peng, 2019 ¹¹⁶	All*	DGF 3y graft survival	0.78 (0.69-0.87)(p<0.0001) 1.06 (1.02-1.11)(p=0.009)
Tingle, 2019 ¹¹⁷	All*	DGF	All: 0.77 (0.67-0.90)(p=0.0006) DCD:0.75(0.64-0.87) (p=0.0002) DBD:0.78(0.65-0.93) (p=0.0006)

Table 7: Risk for delayed graft function, primary nonfunction and graft survival using hypothermic machine perfusion versus static cold storage in metaanalysis during the last decade. DBD, donation after brain death; DCD, donation after circulatory dead; DGF, delayed graft function; ECD, extended criteria donor; NS, not significant; OR, odds ratio; PNF, primary nonfunction; SCS, static cold storage.

* included DBD/ECD/DCD

Organ reconditioning/repair during hypothermic machine perfusion

The main focus of current research to decrease ischemia-reperfusion injury of the kidney during HMP is situated on 4 levels: a) cell therapy (e.g. mesenchymal stem cells), b) drugs and biologicals, c) addition of gas and d) gene therapy.

The constitution of the perfusion solution is likely to play an important role in initial and late graft outcome. Increasing initial ex vivo flow during HMP could be obtained by adding vasodilators in the perfusate as demonstrated by Guarrera during liver preservation.¹¹⁸⁻¹²⁰ Additives in the perfusion solution, such as radical scavengers (e.g. curcumin¹²¹), antioxidants (trimetazidine¹²², cyclodextrin¹²³), .. might decrease the harmful effects of ischemia reperfusion injury.

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Oxygen consumption even under hypothermic conditions, however, continues at approximately 5% - 10% of that at normal body temperature.^{124,125} This fact forms the rationale for investigation into oxygenated HMP techniques. At the moment of the start of this thesis project few evidence was available in preclinical research. Oxygen could potentially increase the production of radical oxygen species and thereby cause increased injury.¹²⁵ However adding oxygen might restore the adenosine triphosphate debt and decrease the harmful effects of ischemia-reperfusion injury. This will be one of the main topics of this PhD manuscript and will be described in detail in the result section.

3. Normothermic machine perfusion

Ex vivo normothermic organ perfusion was introduced in 1934 by Charles Lindbergh and Alexis Carrel, who developed the first machine perfusion to preserve animal organs.¹⁷

During the last decade, there has been a renewed interest into normothermic preservation. In addition to provide oxygenation, normothermic conditions have the advantage of restoring cellular function and metabolism. This gives the opportunity to treat or condition the kidney to target the detrimental effects of ischemia-reperfusion injury or rejection after transplantation. In 2013, M.L. Nicholson and S.A. Hosgood reported the first series of kidney transplants performed after a period of ex vivo normothermic perfusion (Figure 11).¹²⁶

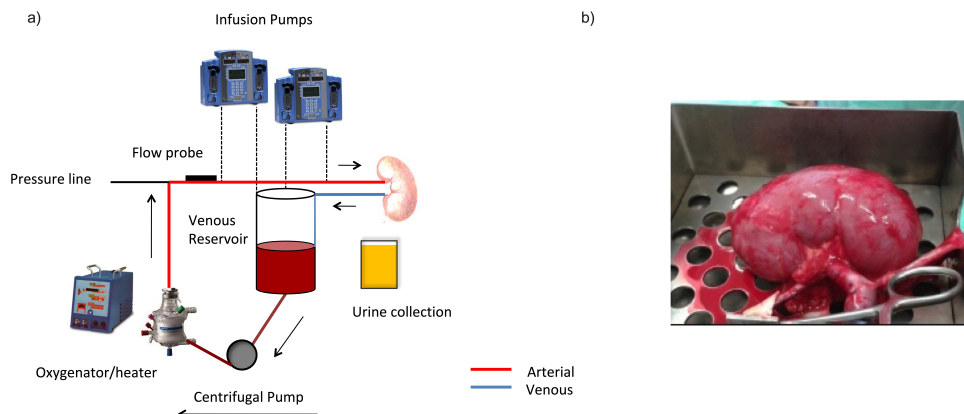


Figure 11: Schematic overview of the ex vivo normothermic perfusion system (a) and an clinical image of an ex vivo normothermic perfused kidney (b) (courtesy by Hosgood SA). The renal artery and vein and ureter were cannulated and the kidney was perfused with 1 unit of compatible packed red blood cells mixed with a priming solution. The kidney was

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perfused at a mean arterial pressure of 60 mmHg and temperature of 35°C for 60 min.

The kidney was perfused after a period of static cold storage for approximately 60 minutes using an normothermic machine perfusion device based on a pediatric cardiopulmonary bypass technology using a red cell-based plasma-free solution. Following perfusion, the kidneys were placed back in static cold storage until transplantation. The results showed a significant reduction in DGF from 36.2% to 5.6% ($p=0.014$). In addition, Hosgood SA et al developed a simple scoring system after 60 minutes of ex vivo normothermic perfusion for pretransplant assessment of kidney quality and acceptability for transplantation.¹²⁷ The assessment score was based on macroscopic assessment, renal blood flow and total urine output.

A multicentre randomised control trial is currently ongoing in the UK. Results are expected in 2020-2021.¹²⁸

Until today, only a few series of initially discarded kidney were assessed by end-ischemic ex vivo normothermic perfusion after static cold storage or HMP and reported to be feasible and a potential strategy to more adequately assess and preserve high-risk kidneys deemed unsuitable for transplantation.^{129,130}

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Personal contributions

I. Aims of the thesis

I. Aims of the thesis

The overall aim was to evaluate the optimal start time, temperature, oxygen concentration and administration technique during ex vivo machine perfusion compared with static cold storage preservation in a porcine ischemia-reperfusion autotransplant model to decrease ischemia-reperfusion injury and subsequently improve early kidney graft function.

Chapter 1 studies the most optimal start time of machine perfusion during kidney preservation. The aim was to determine the most optimal timing to start machine perfusion (continuous from procurement until transplantation versus end-preservation) during kidney preservation and to evaluate the impact of temperature (normothermic versus hypothermic) and oxygen supply (no active oxygen supply versus low dose oxygen supply) during machine perfusion to improve early graft function. Exploring different hypothermic and normothermic machine perfusion strategies combined in one preclinical study was not yet available in literature.

Paper 1: The effect on early renal function of various dynamic preservation strategies in a preclinical pig ischemia-reperfusion autotransplant model. Darius T et al, Am J Transplant. 2019 Mar;19(3):752-762. (Impact factor 7.338).

Chapter 2 studies the optimal perfusate partial oxygen pressure (pO_2) during hypothermic machine perfusion. The aim was to evaluate the impact of different perfusate oxygen concentrations during continuous hypothermic machine perfusion (kidneys pumped from the moment of procurement until transplantation) on metabolic profile of perfusate and tissue samples and on early graft function.

Paper 2: Influence of different partial pressures of oxygen during continuous hypothermic machine perfusion in a pig kidney ischemia-reperfusion autotransplant model. Darius T et al, Transplantation. 2020. Apr;104(4):731-743. (Impact factor 4.74).

Chapter 3 studies the optimal start time and duration for active oxygen administration during hypothermic machine perfusion. The aim was to evaluate if additional oxygen is required during the entire hypothermic machine preservation period to improve mitochondrial protection during cold preservation and subsequently positively influence early graft function. Or is sufficient to add oxygen for a short period at the start or at the end of the machine perfusion to obtain the same effect as compared with continuous oxygenation. This preservation strategy is a new approach in organ preservation and not yet explored. Reducing the duration of oxygen

Personal contributions

I. Aims of the thesis

delivery during hypothermic machine perfusion to a short period either following organ retrieval, or prior to implantation could confer significant logistical advantages. This may be particularly important where airplane travel is required for organ transport.

Paper 3: Brief oxygen uploading during continuous hypothermic machine perfusion is a simple yet effective oxygenation method to improve initial kidney function in a porcine autotransplant model. Darius T et al, Am J Transplant. 2020 Aug;20(8):2030-2043. (Impact factor 7.338).

Chapter 4 studies a new and alternative oxygen administration technique to oxygenate kidney during hypothermic machine perfusion (i.e. direct bubble and surface oxygenation of the perfusate) compared with classical hollow fiber membrane oxygenation. This technique is a new concept to oxygenate kidneys (but also applicable to livers or other organs) and is not yet explored in preclinical and clinical transplantation research. Eliminating the need and handling of a membrane oxygenator as well as the requirement for an oxygen source during transport could keep oxygenated hypothermic machine perfusion simple, facilitates logistics and reduce the total cost of machine perfusion.

Paper 4: Brief bubble and subsequent surface oxygenation is a simple and effective alternative for membrane oxygenation during hypothermic machine perfusion in kidneys. Darius T et al, Transplantation Direct, 2020. 6(7):e571.

Personal contributions
Results: Paper 1

Paper 1: The effect on early renal function of various dynamic preservation strategies in a preclinical pig ischemia-reperfusion autotransplant model.

Personal contributions
Results: Paper 1



ORIGINAL ARTICLE

AJT

The effect on early renal function of various dynamic preservation strategies in a preclinical pig ischemia-reperfusion autotransplant model

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The aims of this study were to determine the most optimal timing to start machine perfusion during kidney preservation to improve early graft function and to evaluate the impact of temperature and oxygen supply during machine perfusion in a porcine ischemia-reperfusion autotransplant model. The left kidney of an approximately 40-kg female Belgian Landrace pig was exposed to 30 minutes of warm ischemia via vascular clamping and randomized to 1 of 6 study groups: (1) 22-hour static cold storage (SCS) (n = 6), (2) 22-hour hypothermic machine perfusion (HMP) (n = 6), (3) 22-hour oxygenated HMP (n = 7), (4) 20-hour HMP plus 2-hour normothermic perfusion (NP) (n = 6), (5) 20-hour SCS plus 2-hour oxygenated HMP (n = 7), and (6) 20-hour SCS plus 2-hour NP (n = 6). Graft recovery measured by serum creatinine level was significantly faster for continuous HMP preservation strategies compared with SCS alone and for all end-ischemic strategies. The active oxygenated 22-hour HMP group demonstrated a significantly faster recovery from early graft function compared with the 22-hour nonactive oxygenated HMP group. Active oxygenation was also found to be an important modulator of a faster increase in renal flow during HMP preservation. Continuous oxygenated HMP applied from the time of kidney procurement until transplant might be the best preservation strategy to improve early graft function.

KEYWORDS

animal models: porcine, delayed graft function (DGF), kidney (native) function/dysfunction, kidney transplantation/nephrology, organ perfusion and preservation, organ procurement and allocation, organ transplantation in general, translational research/science

1 | INTRODUCTION

Kidney transplant is the "gold standard" treatment for end-stage kidney disease, providing superior outcomes in quality of life

and reduced morbidity and mortality compared with dialysis.¹⁻³ A major limitation is the limited number of deceased donor grafts.^{4,5} In response to this organ shortage, kidney grafts from brain death extended criteria donors (ECD)⁶ and from donation

Abbreviations: AST, aspartate aminotransaminase; AUC, area under the curve; DCD, donation after circulatory death; DGF, delayed graft function; ECD, extended criteria donors; HMP, hypothermic machine perfusion; HMPO2, oxygenated hypothermic machine perfusion; HOPE, hypothermic oxygenated perfusion; KPS-1, kidney perfusion solution-1; KT, kidney transplant; LDH, lactate dehydrogenase; LKT, LifePort Kidney Transporter; MMRM, mixed model for repeated measures; NP, normothermic perfusion; PO₂, partial oxygen pressure; ROS, reactive oxygen species; SCS, static cold storage.

after circulatory death (DCD)⁷ are currently accepted for transplant. However, these suboptimal organs result in a higher rate of primary nonfunction, delayed graft function (DGF), and increased rates of early graft loss.⁸⁻¹⁰ DGF is a well-known factor to trigger acute rejection and impairs long-term allograft survival.¹¹

Static cold storage (SCS) alone is the worldwide "gold standard" for organ preservation. During the past 10-15 years, dynamic preservation strategies have been reintroduced as a method of organ preservation with the aim to recondition the kidney during preservation, decrease ischemia-reperfusion injury, and improve early and late graft function.^{12,13} Continuous hypothermic machine perfusion (HMP) started immediately after procurement until implantation demonstrated better transplant outcomes compared with SCS alone¹⁴⁻¹⁶ and reduced the risk of DGF regardless of donor type and cold ischemia time.¹⁷ More recently, end-ischemic strategies have been shown to decrease the incidence of DGF by using HMP¹⁸ or normothermic perfusion (NP)¹⁹ after a period of SCS compared with SCS alone. Preclinical studies have demonstrated a beneficial effect on early graft function by adding oxygen during HMP.²⁰⁻²³ Additionally, continuous NP was explored in a porcine kidney transplant model to evaluate feasibility^{24,25} and demonstrated a positive effect on early graft function.²⁵

Dynamic preservation strategies cover a large spectrum of preservation techniques and often are compared with only SCS alone. Three important variables to be chosen during machine perfusion are the temperature setting, the supplementation of oxygen, and the time to start machine perfusion during the period of the organ

preservation. We designed a comparative preclinical study to evaluate the effect on early graft function of SCS and different dynamic preservation strategies in a pig ischemia-reperfusion autotransplant model. The aims were to determine the most optimal timing to start machine perfusion during kidney preservation to improve early graft function and to evaluate the impact of temperature and oxygen supply during machine perfusion.

2 | MATERIALS AND METHODS

2.1 | Animals

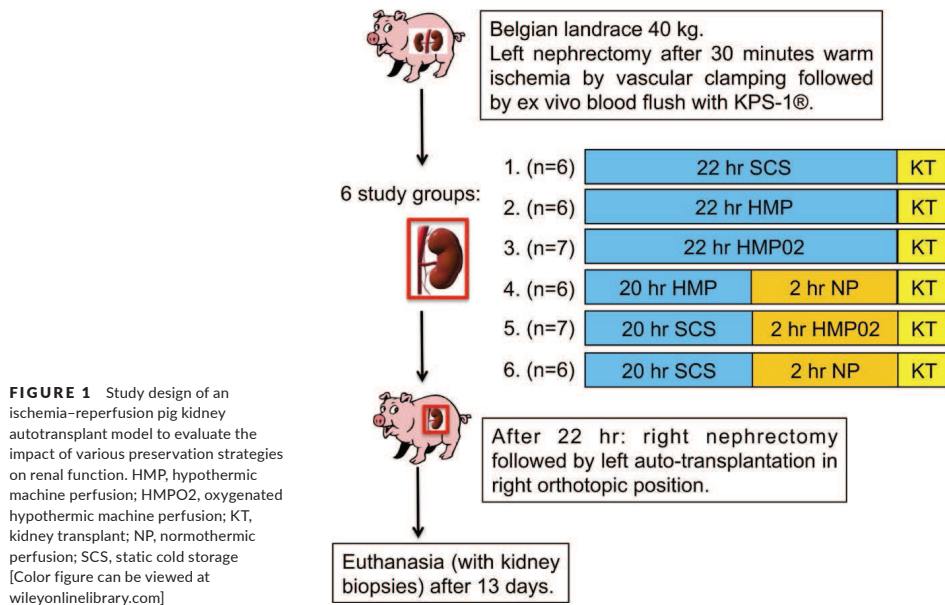
Female Belgian Landrace pigs of approximately 40 kg were used. All experiments were performed according to international guidelines, after approval by the local ethical committee for animal care. The animals were housed and cared according to Belgian laws on experimental animal welfare.

2.2 | Study design

A standardized ischemia-reperfusion pig kidney autotransplant model was used as illustrated in Figure 1.

2.3 | Anesthetic protocol

The anesthetic protocol was identical for the nephrectomy and transplant procedure. Anesthesia was induced via intramuscular injection



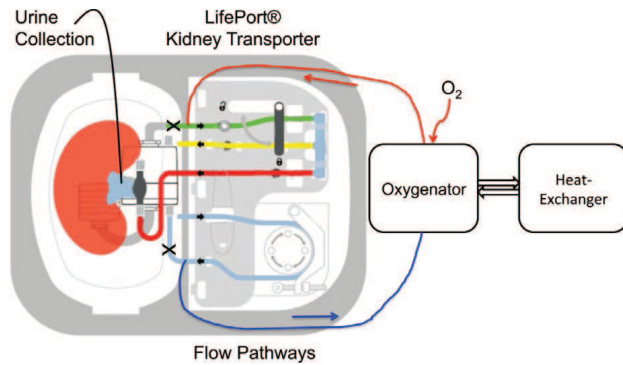


FIGURE 2 Schematic overview of the perfusion set up of the LifePort Kidney Transporter in all oxygenated machine perfusion study groups (courtesy of Organ Recovery Systems, Zaventem, Belgium). An external oxygenator and heat exchanger were added in bypass to the circuit. Only during normothermic perfusion was the ureter of the kidney cannulated for urine collection [Color figure can be viewed at wileyonlinelibrary.com]

of 6 mg/kg Tiletamine 50mg/ml and Zolazepam 50 mg/ml (Virbac, Leuven, Belgium) and 2 mg/kg Xylazine 2% (Bayer, Mechelen, Belgium) and maintained after intubation by 1%-1.5% Isoflurane (Zoetis, Louvain-La-Neuve, Belgium), air and oxygen. Atracurium (GlaxoSmithkline, Waver, Belgium) was administered intravenously as a bolus at 2 mg/kg at the start of the surgical procedure and repeated on indication at 1 mg/kg. Cefazolin (Biopharma, Rome, Italy) 2 g intravenous was given immediately after induction. Sodium chloride 0.9% (Baxter, Lessines, Belgium) at 40 mL/h was administered intravenously during both procedures.

2.4 | Surgical technique

2.4.1 | Left donor nephrectomy

The surgical procedure started with the positioning of a single-lumen vascular access catheter (ref ES-04730; Arrow International, Reading, PA) in the right internal jugular vein and tunneled behind the right ear. A blood transfusion bag containing CPDA (Fresenius Kali, Bad Homburg, Germany) was attached to the central jugular central, and approximately 250 mL of blood was collected and stored at 4°C. Colloid solution (20 mL/kg Geloplasma 3%, Fresenius Kabi, Schelle, Belgium) was used to replace the volume withdrawn in case of hypotension, followed by Hartmann solution 40 mL/kg.

The abdomen was opened through a midline incision. Thirty minutes of warm ischemia was induced by vascular clamping of the renal artery and vein before left kidney removal. An ex vivo blood flush of this kidney was obtained by 200 mL of kidney perfusion solution (KPS-1, Organ Recovery Systems) with 100 cm H₂O. The kidney was stored for a period of approximately 22 hours by 1 of the 6 studied preservation strategies: (a) 22-hour SCS (n = 6), (b) 22-hour HMP (n = 6), (c) 22-hour active oxygenated HMP (HMPO2) (n = 7), (d) 20-hour HMP plus 2-hour NP (n = 6), (e) 20-hour SCS plus 2-hour oxygenated HMP (n = 7), and (f) 20-hour SCS plus 2-hour NP (n = 6) (Figure 1). Abdominal closure was performed.

2.4.2 | Right nephrectomy and autotransplant of the left kidney

The midline wound was reopened, and a right nephrectomy was performed. The left kidney was then autotransplanted into the right renal bed by using the native right renal vein and artery. Furosemide 40 mg (Lasix, Sanofi, Diegem, Belgium) and mannitol 15% (132 mL) (Baxter, Lessines, Belgium) were administered at the start of the anastomosis. An end-to-end ureteroureteroneostomy was performed over a double-J stent. The abdomen was then reclosed.

2.5 | Machine perfusion

The LifePort Kidney Transporter (LKT) (Organ Recovery Systems) was used for all machine perfusion groups. An external oxygenator (Dideco Perfusion Tubing Systems, Sorin Group, Mirandola, Italy) and a heat exchanger were added to the circuit of the LKT (Figure 2). The LKT pumps the perfusate through the kidney vasculature in a pulsatile and pressure-controlled way. The pressure can vary from 0 to 100 mm Hg. The LKT continuously registers renal resistance and flow measures.

2.5.1 | Hypothermic machine perfusion

The KPS-1 solution was pumped through the kidney at a pressure of 30 mm Hg. Oxygen could be added to the perfusate as a function of the study groups (95% O₂/5% CO₂, FiO₂ at 30%) providing a perfusate partial pressure of oxygen (Po₂) of 220-240 mm Hg. Perfusate and kidney were cooled to 3°C.

2.5.2 | Normothermic machine perfusion

Prior to the start NP, the circuit was primed with 250 mL of Ringer solution; 250 mL of autologous whole blood, which was depleted of leukocytes by using a white cell filter (LeukoGuard RS, Haemonetics, Braintree, MA), and heparin 5000 IU. The kidney was connected to the LKT, and the NP solution was pumped through the kidney while adding oxygen to the perfusate (95% O₂/5% CO₂, FiO₂ at 30%) at a

pressure of 30 mm Hg at the start and heat exchanger at 37°C. As soon as the infusion temperature reached 28°C, the pressure was increased to 50 mm Hg and from 32°C to 37°C, the pressure was further increased to 70 mm Hg. The temperature was maintained at 37°C.

Several substances were added to the perfusate as previously described in detail by Nicholson and Hosgood.¹⁹

2.6 | Postoperative care

Buprenorphine hydrochloride 0.01 mg/kg intravenous was administered after left nephrectomy and up to 48 hours after transplant for postoperative analgesia, Hartmann solution (40 mL/kg/24 h) during the first 2 days after transplant and long-acting oxytetracycline 20 mg/kg intramuscular 2 days after the transplant. Animals were allowed free access to water immediately, and food was introduced on the first day after transplant.

The surviving animals were killed on the 13th day after transplant by a lethal intravenous injection of T61 (Intervet International, MDS Animal Health, Boxmeer, Netherlands) under general anesthesia.

2.7 | Outcome measures

The primary endpoint was early graft function (measured as daily serum creatinine). The secondary endpoints were renal flow and resistance during machine perfusion and perfusate and serum biomarkers.

2.8 | Statistical methods

The trial followed a parallel design with 6 active groups. In total, 46 animals were included in this analysis. No explicit imputation was foreseen, so all analyses were conducted on the observed cases only.

The primary endpoint of early graft function was assessed through analysis of serum creatinine over time by using GLM techniques. Serum creatinine values were normalized to 40 kg animal body weight before use in the analysis to account for differences

due to animal/organ size. Serum creatinine was analyzed by using a longitudinal mixed model for repeated measurements (MMRM) with main factors of treatment, time-point, and interaction term to test for overall difference in graft function over time between treatment groups. Individual comparison of groups were conducted using contrast analyses within the main MMRM model testing the null hypothesis that the difference in least-squares means between 2 groups under investigation was 0 (no mean difference between groups). For the contrast analysis, *P* values (based on *t* test of least squares means) were reported along with a graphic mean (SE) plot where deemed appropriate. All secondary endpoint variables were similarly modeled to the primary endpoint. In case of variables without longitudinal structure (eg, area under the curve [AUC]), the MMRM model was reduced to a basis ANOVA model with factor treatment. As this was a pre-clinical exploratory trial, no formal sample size calculations were performed and no correction for multiplicity was foreseen. All analyses were performed by using SAS 9.2 or SAS 9.4 (SAS Institute, Cary, NC).

3 | RESULTS

3.1 | Operative data, major complications, and animal survival

Operative data are shown in Table 1. Forty-six autotransplants were performed. Eight were excluded from the analysis for death in relation to complications (sudden death [*n* = 3], bleeding from central line damage [*n* = 1], necrotizing anal prolapse [*n* = 1], arterial thrombosis [*n* = 1], intestinal volvulus [*n* = 1], and massive bleeding after reperfusion [*n* = 1]).

3.2 | Graft function

3.2.1 | Impact of timing to start machine perfusion

An area under the curve (AUC) analysis of the serum creatinine from day 1 until day 13 after transplant is shown in Figure 3A.

TABLE 1 Operative data for 38 pigs in an ischemia-reperfusion autotransplant model according to the study group

Variable	22 h SCS (n = 6)	22 h HMP (n = 6)	22 h HMPO2 (n = 7)	20 h HMP +2 h NP (n = 6)	20 h SCS +2 h HMPO2 (n = 7)	20 h SCS +2 h NP (n = 6)
Weight, kg	40.40 ± 5.57	39.23 ± 2.00	43.26 ± 3.14	41.96 ± 6.91	39.96 ± 5.94	41.30 ± 3.43
Left nephrectomy procedure time, h:min	3:41 ± 0:46	3:16 ± 0:31	3:27 ± 0:44	3:16 ± 0:43	3:37 ± 0:23	3:49 ± 0:13
Right nephrectomy and autotransplant procedure time, h:min	3:04 ± 0:38	3:52 ± 0:54	3:36 ± 0:58	3:07 ± 0:42	3:38 ± 0:45	3:22 ± 0:34
Cold ischemic time, h:min	21:56 ± 0:37	22:40 ± 0:36	22:23 ± 0:24	20:51 ± 0:57	22:14 ± 0:24	20:50 ± 0:52
Anastomotic time, min	30 ± 5	32 ± 4	30 ± 6	26 ± 7	28 ± 4	30 ± 7
Total ischemic time, h:min	22:56 ± 0:35	23:42 ± 0:35	23:23 ± 0:26	21:47 ± 0:51	23:21 ± 0:29	21:48 ± 0:50

HMP, hypothermic machine perfusion; HMPO2, oxygenated hypothermic machine perfusion; NP, normothermic perfusion; SCS, static cold storage. Data are given as the mean ± SD.

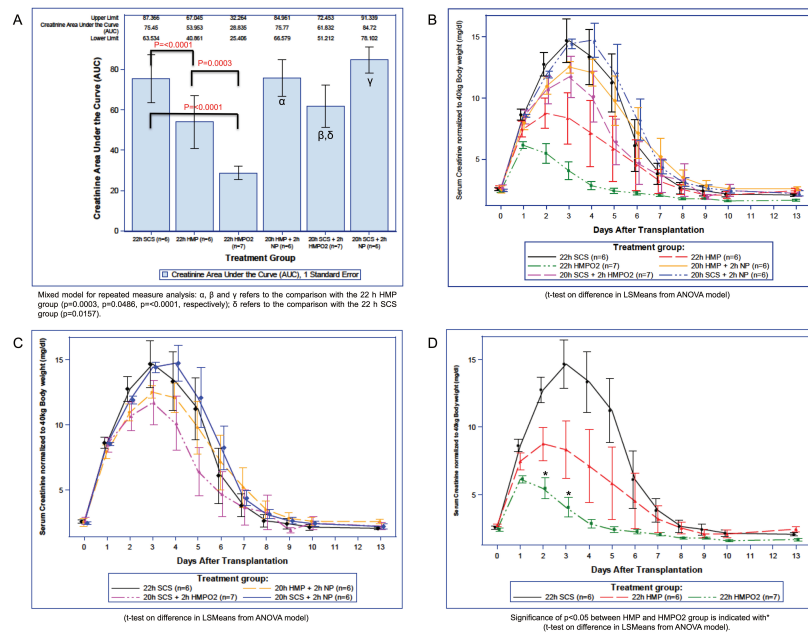


FIGURE 3 Area under the curve analysis of serum creatinine from day 1 to day 13 after transplant according to the study group demonstrating that continuous HMP with or without O_2 significantly improved early graft function compared with SCS alone and all end-ischemic machine perfusion strategies (A). Evolution of daily postoperative serum creatinine in all study groups (B), in end-ischemic machine perfusion groups (C), and continuous HMP groups with or without oxygen compared with the SCS group (D). HMP, hypothermic machine perfusion; HMP02, oxygenated hypothermic machine perfusion; NP, normothermic perfusion; SCS, static cold storage

Comparison of the study groups demonstrated a significant difference in favor of both 22-hour continuous HMP groups, independent of the supplementation of oxygen, compared with SCS alone ($P < .0001$) and compared with all end-ischemic machine perfusion groups.

The daily evolution of serum creatinine after transplant is shown in Figure 3B. Peak serum creatinine levels in end-ischemic strategies (including NP and hypothermic oxygenated perfusion [HOPE]) did not significantly differ from SCS alone. Continuous HMP with or without active oxygen supply demonstrated a more effective strategy to decrease serum creatinine during the first 7 days after transplant compared with SCS alone and compared with all end-ischemic strategies.

3.2.2 | Impact of oxygen and temperature on end-ischemic machine perfusion strategies

Impact of temperature: normothermic perfusion after SCS or HMP: Both study groups using end-ischemic NP did not demonstrate a significant difference in serum creatinine AUC (Figure 3A) or in daily serum creatinine (Figure 3C) compared with the SCS-alone group.

Impact of oxygen: use of HOPE end-ischemic: Serum creatinine AUC using HMP02 after SCS was significant lower compared with the SCS alone group ($P = .0157$) (Figure 3A). No significant

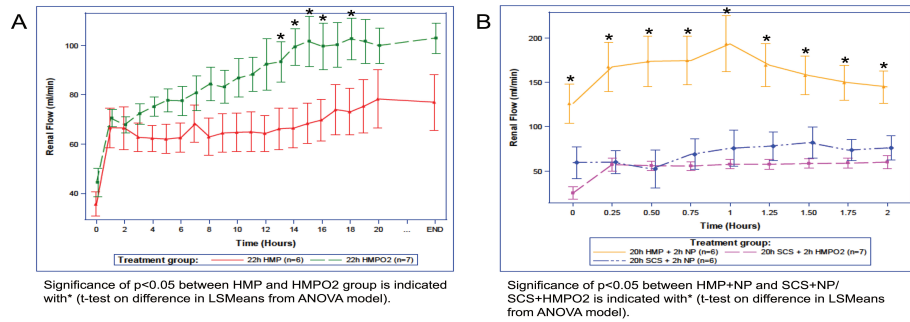


FIGURE 4 Influence of oxygen supply on ex vivo renal flow during continuous hypothermic machine perfusion with or without active oxygenation (A) and during end-ischemic machine perfusion groups (B). HMP, hypothermic machine perfusion; HMPO2, oxygenated hypothermic machine perfusion; NP, normothermic perfusion; SCS, static cold storage

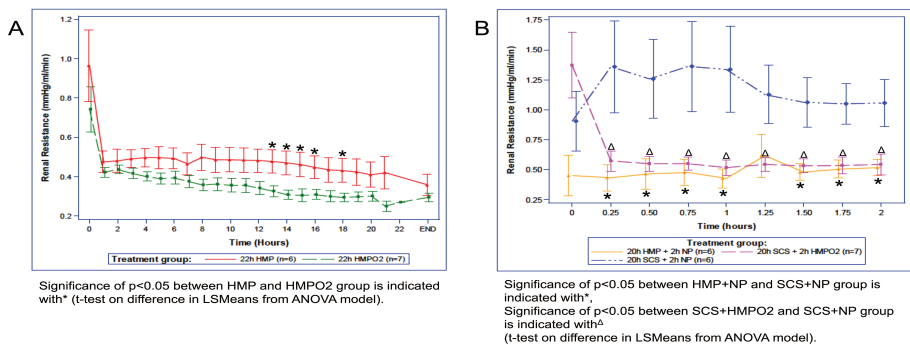


FIGURE 5 Evolution of renal resistance in the continuous hypothermic machine perfusion groups with or without oxygenation (A) and in the 3 end-ischemic machine perfusion groups (B). HMP, hypothermic machine perfusion; HMPO2, oxygenated hypothermic machine perfusion; NP, normothermic perfusion; SCS, static cold storage

difference in daily serum creatinine was observed at days 1, 2, 3, 7, and 13 after transplant between both study groups (Figure 3C).

3.2.3 | Impact of oxygen during continuous HMP

Comparison of the study groups demonstrated a significant difference of serum creatinine AUC in favor of the continuous HMPO2 group compared with both SCS alone ($P < .0001$) and nonactive oxygenated HMP groups ($P = .0003$) overall (Figure 3A).

The daily evolution of serum creatinine after transplant is shown in Figure 3D for the continuous dynamic preservation strategies compared with SCS. The serum creatinine was significantly lower at day 1 after transplant using continuous HMPO2 compared with SCS alone ($P = .0005$). No difference was observed at day 1 between the non-oxygenated HMP group and the SCS alone group ($P = .0903$), nor between the nonactive oxygenated and the HMPO2 group ($P = .0507$). However, at days 2 and 3 after transplant, the serum creatinine was significantly lower in both the 22-hour oxygenated group (day 2:

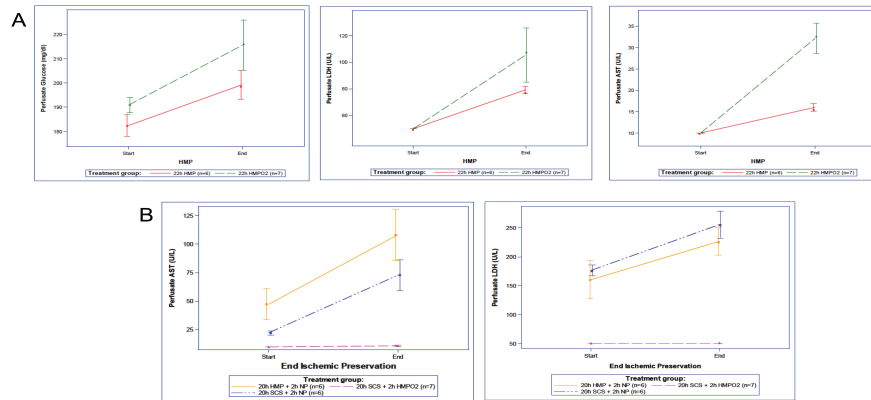


FIGURE 6 Evolution of perfusate glucose, LDH and AST during continuous hypothermic machine perfusion with and without active oxygenation (A) and evolution of perfusate LDH and AST in the 3 end-ischemic machine perfusion groups (B). AST, aspartate aminotransaminase; HMP, hypothermic machine perfusion; HMPO2, oxygenated hypothermic machine perfusion; LDH, lactate dehydrogenase; NP, normothermic perfusion; SCS, static cold storage

$P < .0001$; day 3: $P < .0001$) and the nonoxygenated HMP group (day2: $P = .0045$; day 3: $P = .0033$) group compared with SCS alone.

At days 2 and 3, a significant difference was observed in serum creatinine in favor of the HMPO2 group compared with the nonoxygenated HMP group ($P = .0138$ and $P = .0334$ at days 2 and 3, respectively). From day 7, no difference was observed between the study groups.

3.3 | Impact of oxygen on renal flow and resistance during machine perfusion

3.3.1 | Impact of oxygen during continuous HMP

Active oxygenation during continuous HMPO2 resulted in faster increase in renal flow compared with nonactive oxygenated continuous HMP (Figure 4A). The difference in renal flow was significantly higher in favor of the oxygenated HMP group starting at 13 hours of HMP ($P = .0469$) until 18 hours of machine perfusion ($P = .0433$). Thereafter, no significant differences in renal flow were observed between both groups ($P = .0600$) at the end of the machine perfusion. The same observation was noted for renal resistance in favor of the HMPO2 group (Figure 5A).

3.3.2 | Impact of oxygen during end-ischemic strategies

End-ischemic NP after previous HMP resulted in a significantly higher renal flow from the start until the end of the machine

perfusion compared with NP after SCS ($P \leq .0001$) (Figure 4B). No difference was observed in renal flow between end-ischemic NP and HMPO2 after 20 hours of SCS ($P = .3024$). The same findings were also observed for renal resistance in favor of the end-ischemic NP after previous HMP (Figure 5B).

3.4 | Perfusate parameters

No difference was found in perfusate glucose, lactate dehydrogenase (LDH), and aspartate aminotransaminase (AST) at start and at the end between the oxygenated and nonoxygenated continuous HMP group (Figure 6A).

End-ischemic HMPO2 after a period of SCS did not influence perfusate AST and LDH (Figure 6B). Perfusate AST at start of NP was more pronounced after preceding HMP compared with preceding SCS ($P = .0134$) but did not reach statistical significance at the end of NP ($P = .0500$). In contrast, perfusate LDH was higher at the start and end of NP after preceding SCS compared with preceding HMP but did not reach statistical significance.

3.5 | Evolution of postoperative serum LDH and AST

A postoperative LDH/AST peak was observed at day 1 but dropped significantly after day 3. No significant difference was observed in the postoperative LDH/AST profile from day 1 to 13 between all groups (Figure 7A-D).

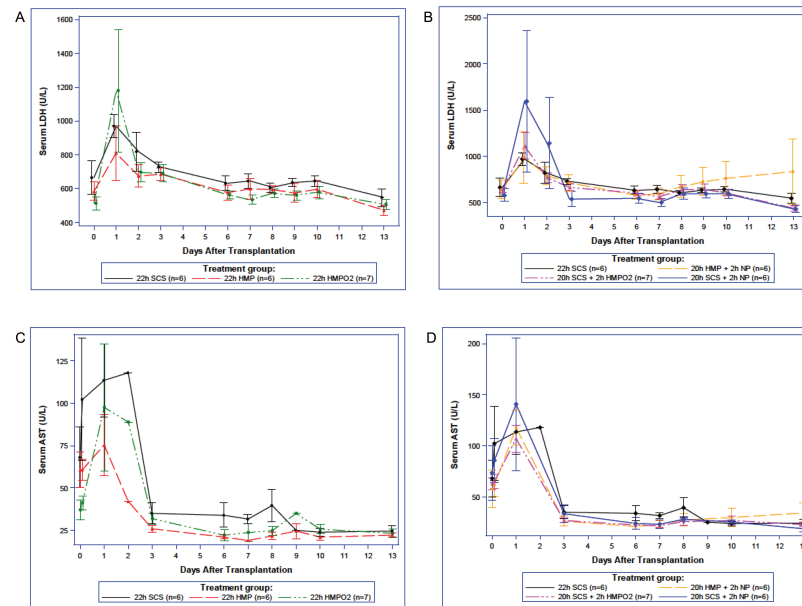


FIGURE 7 Evolution of posttransplant serum LDH and AST in the continuous HMP groups with or without oxygen and the SCS group (A and C, respectively) and in the 3 end-ischemic machine perfusion and SCS groups ([B] and [C], respectively). AST, aspartate aminotransaminase; HMP, hypothermic machine perfusion; HMP+O2, oxygenated hypothermic machine perfusion; LDH, lactate dehydrogenase; NP, normothermic perfusion; SCS, static cold storage

4 | DISCUSSION

This ischemia–reperfusion porcine kidney autotransplant model is the first animal study on early renal function that compares different continuous, end-ischemic, and SCS preservation strategies with the aim to determine the machine perfusion strategy that offers the best graft outcome. Hypothermic perfusion strategies applied from time of kidney procurement until transplant and regardless of supplemental oxygenation led to a significantly positive effect on early graft function compared with SCS alone and compared with different end-ischemic strategies (including HOPE and NP). The overall treatment effect on posttransplant serum creatinine of each preservation strategy demonstrated a significant difference in favor of the continuously oxygenated HMP group compared with the nonactive oxygenated HMP group.

The active oxygenated 22-hour HMP group demonstrated a significantly faster recovery from early graft function compared with the 22-hour nonactive oxygenated HMP group. Continuous active

oxygenated HMP is probably the most convenient to apply in clinical setting for organ sharing. Active oxygenation seems an important modulator for a faster increase in renal flow during HMP preservation. This effect on early graft function was obtained during continuous HMP but not when applied as an end-ischemic strategy. This result is in contrast to a recent study in a rat model demonstrating a benefit of end-ischemic oxygenated HMP after SCS.²³ However, our results are in line with a preclinical study that demonstrated the benefit of active oxygen supplementation during HMP in a porcine model with a partial oxygen pressure of approximately 760–800 mm Hg.²¹

End-ischemic NP demonstrated no positive effect on early graft function in this study. In 2013, the first clinical study, applying end-ischemic NP after SCS (using kidney grafts originated from ECD) demonstrated a reduction of the DGF rate in favor of the NP group (5.6% vs 36.2%, $P = .014$).¹⁹ Despite using the same perfusate composition, we observed a higher serum creatinine at day 3 after transplant compared with SCS alone, illustrating that NP after SCS could lead to an even more substantial ischemia–reperfusion injury.

It could be speculated that the kidneys used in the clinical trial were more damaged organs and therefore may benefit more from the normothermic reconditioning than the kidneys used in our model, which originated from healthy pigs with 30 minutes of warm ischemia by vascular clamping. In line with previous published work by Hosgood, our study demonstrates that the use of NP after a period of HMP did not improve early graft recovery.²⁶ We agree that NP remains an interesting tool to evaluate transplantability of discarded kidneys^{27,28} and more insights toward the precise role of NP will be obtained by an ongoing multicenter randomized clinical trial.²⁹

Kaths et al demonstrates that intermediate (8 hours) or prolonged (16 hours) NP following SCS is superior to brief preimplantation NP in a pig kidney transplant model.^{25,30} They suggest that 1 hour of NP is insufficient time for effective repair mechanisms and results in an upregulation of proinflammatory signaling by rapid warming. These results are in line with our results but in contrast with the results from Hosgood and colleagues.^{19,31} Underlying mechanisms of NP are not fully understood. Different perfusion systems in combination with differences in perfusate composition, use of vasodilator, pressure settings, flow rates and the duration of the machine perfusion are probably an explanation for these contrasting results. We are aware that our study lacks a continuous NP group; however, because of its complexity, and the fact that the NP protocol used in our study was not designed for long periods, we did not include this in our study design.

Our study demonstrated a significantly faster recovery from early graft function using end-ischemic HMPO2 after SCS compared with SCS alone. This strategy could be an interesting tool to decrease the DGF rate, especially in transplant programs dealing with a high incidence of DGF. Consistently, the strategy without active oxygenation has been recently confirmed in clinics by the Essen Group demonstrating a faster recovery from early graft function using end-ischemic nonactive oxygenated HMP for at least 2 hours after SCS compared with SCS alone.¹⁸ In a rodent model reported by Kron et al, the end-ischemic HMPO2 compared with other clinically (SCS alone and end-ischemic normothermic perfusion) used preservation approaches was demonstrated to be a superior technique.²³ However, it should be stressed that in the latter study, the perfusate oxygen concentration was higher than in our protocol (450–600 vs 220–240 mm Hg).

Historically, mitochondrial reactive oxygen species (ROS) production during ischemia–reperfusion injury has been considered to be a nonspecific response to reperfusion. However, a recent metabolomic analysis by Chouchani suggests that selective accumulation of the citric acid cycle intermediate succinate is a universal metabolic signature of ischemia in a range of tissues and is responsible for mitochondrial ROS production during reperfusion.^{32,33} On reperfusion, the accumulated succinate is rapidly re-oxidized by succinate dehydrogenase, driving extensive ROS generation by reverse electron transport at mitochondrial complex I. Superoxide generation by reverse electron transport upon reperfusion is the major source of ischemia–reperfusion injury. Kron et al demonstrated in a rodent kidney transplant model that end-ischemic hypothermic oxygenated

perfusion did not lead to a relevant ROS release, in contrast to end-ischemic NP.²³ The proposed working mechanism of oxygenated HMP is situated on 3 levels. First, active oxygenation during cold perfusion influences the metabolic status. It improves the function of the mitochondria enzyme complexes probably leading to forward instead of reverse electron flow therefore uploading adenine nucleotides (ATP/ADP) during cold oxygenation. Cellular redox state changes from reduced to oxidized and also succinate is also removed. Second, it affects the endothelium by cleaning the glycocalyx and upregulation of shear stress-sensitive genes. Third, it will lead to activation of protective pathways (eg, hypoxia inducible factors, heat shock protein 70, etc.). These changes during HMPO2 will lead to less initial ROS, and the amount of released danger-associated molecular patterns will be subsequently decreased, resulting in less inflammation and a decreased ischemic injury after reperfusion.

Currently, perfusion characteristics during HMP, such as renal resistance, have a limited value for prediction of delayed graft function.^{17,34} Few data are available regarding the impact of oxygen on perfusion characteristics during HMP. Our data demonstrate a faster increase in renal perfusion flow and a lower renal resistance when adding active oxygen to the perfusate during long periods of HMP compared with nonactive oxygenated HMP. A possible explanation might be the crucial role of nitric oxide as a potent vasodilator during perfusion. The synthesis of nitric oxide is the result from an oxidation process and this complex reaction requires the presence of co-substrate oxygen.³⁵ Because the variance is much lower for renal resistance compared with renal flow during machine perfusion we believe that renal resistance is a more reliable parameter than renal flow. These results are in line with those of Hoyer et al, which also demonstrate a faster increase in perfusion flow comparing 21-hour HMP with air and with 100% oxygen.²¹ Most studies, however, compare only the perfusion characteristics at the start and at the end but not the trend during machine perfusion.³⁶ Further research is needed to examine their value in graft quality assessment and the determination of optimal perfusion time.

Based on our preclinical results, kidney preservation and reconditioning should be started immediately after ex vivo blood flush and maintained on HMP conditions during the whole period of organ preservation. Active oxygenation during HMP seems to be an extremely important factor for a faster increase in ex vivo renal flow during machine perfusion and to have a positive influence on early graft function. The influence of different partial oxygen pressures and optimal timing to start active perfusate oxygenation during HMP (initial at donor site, continuous, or end-ischemic at receptor site) should be confirmed by clinical studies in the near future.

Translation of our results into the clinical setting must be done with caution. We chose 30 minutes of preceding warm ischemia before procurement in this autotransplant model. First, 30 minutes of warm ischemia time is similar to the mean first warm ischemia time (time from switch of ventilation to start cold perfusion) in most clinical controlled DCD programs worldwide. Second, 30 minutes is frequently used in preclinical models.^{21,25,26} Third, longer warm ischemia time results in a higher degree of renal insufficiency after

autotransplant and was badly tolerated by the pigs and therefore declined because of ethical reasons. Because most extended deceased donor kidneys have an inferior quality compared with our studied pig kidneys, there might be even more benefit from the reconditioning of continuous active oxygenated HMP. In contrast to the cardiopulmonary bypass (flow-driven) technology used by Hosgood,¹⁹ the LKT has a roller pump (pressure-driven), originally designed to perfuse kidneys in hypothermic conditions. To reduce hemolysis of the diluted blood, NP in our model was limited to 2 hours. Therefore, we consider it unlikely that this could have influenced our primary endpoint, but comparison with other NP types must be done with caution.

In conclusion, continuous oxygenated HMP applied from the time of kidney procurement until transplant might be the best preservation strategy to improve early graft function compared with SCS alone or continuous nonactive oxygenated HMP and compared with different end-ischemic preservation strategies.

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DISCLOSURE

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. The LifePort Kidney Transporter, the Perfusion Circuits and the oxygenators were supplied by Organ Recovery Systems (Zaventem, Belgium).

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Summary Paper 1

Aim of the study:

The aims of the study were to determine the most optimal timing to start machine perfusion during kidney preservation to improve early kidney graft function and to evaluate the temperature and oxygen supply during machine perfusion in a porcine ischemia-reperfusion DCD autotransplant model.

Main conclusions:

- Continuous hypothermic machine perfusion (HMP) from procurement until transplantation was significantly better than static cold storage alone and end-ischemic machine perfusion strategies to improve early graft function.
- Continuous oxygenated HMP (30% carbogen (95% O₂ / 5% CO₂)) further enhanced renal recovery.
- Active oxygenation was found to be an important modulator of a faster increase in renal flow during HMP preservation. This results in a higher oxygen delivery to the tissue during machine perfusion.
- No differences were observed between perfusate AST and LDH (both markers of ischemia reperfusion injury) at the end of the machine perfusion between all groups.
- No differences in serum AST and LDH were observed between all study groups during the follow-up.

Personal contributions
Results: Paper 2

Paper 2: Influence of different partial pressures of oxygen during continuous hypothermic machine perfusion in a pig kidney ischemia-reperfusion autotransplant model.

Personal contributions
Results: Paper 2



Influence of Different Partial Pressures of Oxygen During Continuous Hypothermic Machine Perfusion in a Pig Kidney Ischemia-reperfusion Autotransplant Model

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Background. The optimal perfusate partial pressure of oxygen (P_{O_2}) during hypothermic machine perfusion (HMP) is unknown. The aims of the study were to determine the functional, metabolic, structural, and flow dynamic effects of low and high perfusate P_{O_2} during continuous HMP in a pig kidney ischemia-reperfusion autotransplant model. **Methods.** The left kidneys of a ± 40 kg pigs were exposed to 30 minutes of warm ischemia and randomized to receive 22-hour HMP with either low perfusate P_{O_2} (30% oxygen, low oxygenated HMP [HMPO₂]) ($n = 8$) or high perfusate P_{O_2} (90% oxygen, HMPO₂high) ($n = 8$), before autotransplantation. Kidneys stored in 22-hour standard HMP ($n = 6$) and 22-hour static cold storage ($n = 6$) conditions served as controls. The follow-up after autotransplantation was 13 days. **Results.** High P_{O_2} resulted in a 3- and 10-fold increase in perfusate P_{O_2} compared with low HMPO₂ and standard HMP, respectively. Both HMPO₂ groups were associated with superior graft recovery compared with the control groups. Oxygenation was associated with a more rapid and sustained decrease in renal resistance. While there was no difference in functional outcomes between both HMPO₂ groups, there were clear metabolic differences with an inverse correlation between oxygen provision and the concentration of major central metabolites in the perfusion fluid but no differences were observed by oxidative stress and metabolic evaluation on preimplantation biopsies. **Conclusions.** While this animal study does not demonstrate any advantages for early graft function for high perfusate P_{O_2} , compared with low perfusate P_{O_2} , perfusate metabolic profile analysis suggests that aerobic mechanism is better supported under high perfusate P_{O_2} conditions.

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INTRODUCTION

Machine preservation of kidneys during the organ preservation period is a subject of great clinical interest. More effective perfusion strategies could minimize the harmful

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effects of ischemia-reperfusion injury, hence improving both early and late allograft function. This is relevant in an era of high-risk donor organs, which are associated with increased rates of primary nonfunction, delayed graft function, and increased rates of early graft loss.¹

The optimal perfusion condition for kidneys has not yet been established. Important perfusion variables, such as storage temperature, active oxygen supplementation, perfusion fluid composition, and start time of perfusion (initial, continuous, or end ischemic), are under scrutiny. Our group has recently compared various normothermic and hypothermic preservation techniques in a porcine ischemia-reperfusion model.² Continuous oxygenated hypothermic machine perfusion (HMPO₂) applied from the time of kidney procurement until transplant demonstrated to be the best preservation strategy to improve early graft function compared with static cold storage (SCS) alone or continuous standard HMP and compared with different end-ischemic preservation strategies (including hypothermic oxygenated perfusion and normothermic perfusion).² Although several preclinical studies have similarly shown a beneficial effect of supplemental oxygenation (continuous or end ischemic) for early graft function,³⁻⁷ there is evidence signifying the promotion of aerobic metabolism and restoration of cellular ATP only occurs at higher oxygen tension.^{8,9}

The optimal perfusate partial pressure of oxygen (PO₂) during HMP is unknown. We therefore aim to evaluate

the impact of different perfusate oxygen concentrations during continuous HMP on early graft function, tubular function, serum biomarkers, renal flow, resistance and PO₂ during machine perfusion, perfusate metabolic profile and oxidative stress, metabolic evaluation, and graft histology of preimplantation biopsies in a pig kidney ischemia-reperfusion autotransplant model.

MATERIALS AND METHODS

Animals

Female Belgian Landrace pigs of approximately 40 kg were used. All experiments were performed according to international guidelines after approval by the local Ethical Committee for Animal Care. The animals were housed and cared according to Belgian laws on experimental animal welfare.

Study Design

A standardized ischemia-reperfusion pig kidney autotransplant model was used (Figure 1).

Anesthetic and Surgical Protocol

Both protocols were already described in detail before.² Briefly, 30 minutes warm ischemia was induced in the left kidney by vascular clamping of the renal vessels. Thereafter, the kidney was removed and flushed with 200 mL of

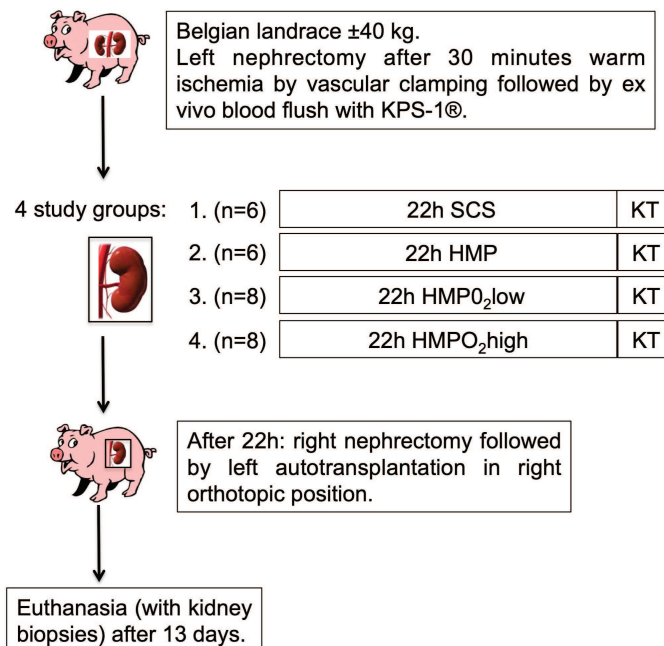


FIGURE 1. Study design of an ischemia-reperfusion pig kidney autotransplant model to evaluate the impact of different oxygen pressures during continuous hypothermic machine perfusion on early graft function. HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion; KPS-1, kidney perfusion solution-1; KT, kidney transplantation; SCS, static cold storage.

Kidney Perfusion Solution (KPS-1, Organ Recovery Systems, Diegem, Belgium) at 4°C at a hydrostatic pressure of 100 cmH₂O. The kidney was stored for a period of approximately 22 hours by 1 of the 4 studied preservation strategies: (1) 22-hour SCS (n = 6), (2) 22-hour standard (no active oxygen supply) HMP (n = 6), (3) 22-hour active HMPO₂ (30% oxygen, HMPO₂low) (n = 8), and (4) 22-hour active HMPO₂ (90% oxygen, high oxygenated HMP [HMPO₂high]) (n = 8) (Figure 1). Following preservation, the left kidney was autotransplanted into the right renal bed after a right nephrectomy. Postoperative care was identical as previously described.²

The surviving animals were euthanized on the 13th day after transplantation by a lethal intravenous injection of T61 (Intervet International, MDS Animal Health, Boxmeer, The Netherlands) under general anesthesia after surgical biopsies of the transplanted kidney.

Machine Perfusion

The LifePort Kidney Transporter (Organ Recovery Systems, Diegem, Belgium) was used for all machine perfusion groups. The circuit was modified to include an external oxygenator (Dideco Perfusion Tubing Systems, Sorin Group, Mirandola, Italy) and heat exchanger in both active oxygenated groups. The KPS-1 solution was pumped through the kidney at a pressure of 30 mmHg. Carbogen (95% O₂/5% CO₂) was used for oxygen supplementation at either 30% or 90% in the low- and high-oxygenated group, respectively. The temperature of kidney and circulating perfusate was maintained at 2°C–8°C.

Outcome Measures

The primary endpoint was early graft function (measured as daily serum creatinine). The secondary endpoints were tubular function (measured by fractional excretion of sodium and protein creatinine ratio in the urine), serum biomarkers (lactate dehydrogenase [LDH]) and aspartate transaminase [AST]), renal flow and resistance during machine perfusion, perfusate Po₂ levels, perfusate metabolic profile and oxidative stress, metabolic evaluation, and graft histology on preimplantation biopsy.

Analyses

Renal Flow and Resistance Measurement During HMP and Perfusate Analyses

The machine perfusion device continuously registers renal flow and resistance. Perfusate samples were taken at start and at the end of the machine perfusion. Perfusate Po₂ was measured at start and end of all machine perfusion groups using the I-STATS1 (Abbott Point of Care Inc, Princeton, NJ). Glucose, AST, and LDH analysis on perfusate were performed by a dry chemistry analyzer Fui Dri-Chem NX500 (Fujifilm Corporation, Tokyo, Japan). For metabolite identification and quantification using nuclear magnetic resonance (NMR) spectroscopy, neat perfusate samples were mixed with a concentrated NMR buffer, granting end concentrations of 0.5 mmol/L 4,4-dimethyl-4-silapentane-1-sulfonic acid, 0.1 M phosphate buffer, 25% deuterium oxide, and 2 mmol/L imidazole for each sample. Proton NMR (¹H-NMR) spectra were acquired on a 600-MHz Bruker Avance III NMR spectrometer in 5 mm NMR tubes. The spectra were phase and baseline corrected

with Metabolab software.¹⁰ Metabolite identification and quantification were performed using Chenomx NMR Suite (Chenomx, Edmonton, AB, Canada). The endpoint perfusate metabolic profile was assessed, with acetate, adenine, alanine, aspartate, formate, gluconate, glutamate, glutathione, glycine, hypoxanthine, isoleucine, lactate, leucine, mannitol, myoinositol, and succinate quantified.

Blood and Urine Analysis

Venous blood samples were taken daily during follow-up. Urine samples were collected for biochemical analysis. Blood and urine analysis (eg, creatinine, urea, sodium, glucose, AST, LDH, proteinuria) were determined with methods of the clinical routine.

Tissue Sampling and Analysis

Wedge biopsies were taken at 3 time-points. First, baseline samples were taken before the onset of preservation after ex vivo blood flush with KPS-1. A second preimplantation sample was taken after 22 hours of preservation with a third endpoint sample taken before euthanasia. Biopsies were stored fresh at –80°C until metabolic analysis and also fixed in acidified formal alcohol, dehydrated, and embedded in paraffin wax for histology. Protein levels were determined by Western blotting using preimplantation samples of frozen kidney biopsies as previously described.¹¹ Levels of oxidative enzymes such as nicotinamide adenine dinucleotide phosphate oxidase (NADPH) 2 and 4 (NADPH oxidase 2 [NOX2] and NADPH oxidase 4 [NOX4]) (Abcam ab31092, Lifespan Biosciences, LS-C313066), heme oxygenase 1 (HO-1) (Abcam ab13243), antioxidant enzymes such as superoxide dismutase 1 (SOD1) (Abcam ab13498), and peroxiredoxin 1 and reactive oxygen species-sensitive protein such as hypoxia-inducible factor 1α (HIF-1α) (BD transduction, BD 610959), have been detected and normalized using the levels of β actin. Metabolites (lactate, succinate, glutamate, AMP, ADP, ATP, and oxidized and reduced nicotinamide adenine dinucleotide [NADH]) were extracted from frozen kidney biopsies by methanol-chloroform extraction and analyzed by liquid chromatography electrospray ionization mass spectrometry based on the description by Coulier et al.¹² Metabolite quantification was performed as described by Veiga-da-Cunha et al.¹³

Sections of 4 μm were cut and stained with hematoxylin and eosin for evaluation using light microscopy. Sections were scored blindly assessing changes according to the histopathologic consensus criteria for preimplantation kidney biopsies¹⁴ and an acute tubular injury score described by Hosgood et al¹⁵ assessing changes in 4 morphological variables: tubular dilatation, tubular debris, vacuolation, and infiltration.

Statistical Methods

The trial follows a parallel design with 4 active groups. No explicit imputation was foreseen, so all analyses were conducted on the observed cases only.

The primary endpoint of early graft function was assessed through analysis of serum creatinine over time using generalized linear model techniques. Serum creatinine values were normalized to 40 kg animal body weight before use in the analysis to account for differences due

TABLE 1.**Operative data for 28 pigs in an ischemia-reperfusion autotransplant model according to the study group**

Variable	22 h SCS (n = 6)	22 h HMP (n = 6)	22 h HMPO _{low} (n = 8)	22 h HMPO _{high} (n = 8)	P
Weight pig, kg	40.40 ± 5.57	39.23 ± 2.00	43.29 ± 2.91	43.83 ± 7.30	0.288
Left nephrectomy procedure time, h:m	3:41 ± 0:46	3:16 ± 0:31	3:31 ± 0:42	3:31 ± 0:26	0.719
Weight left kidney, mg					0.084
Before preservation	89 ± 10	100 ± 13	104 ± 14	109 ± 18	
After preservation	80 ± 9	151 ± 22	154 ± 22	149 ± 30	
Perfusate P _{O₂} , mm Hg (measured at 37°C)					
At start HMP	—	170 ± 4	217 ± 9	726 ± 41	<0.0001
At end HMP	—	70 ± 5	222 ± 8	718 ± 40	<0.0001
Right nephrectomy and autotransplant procedure time, h:m	3:04 ± 0:38	3:52 ± 0:54	3:31 ± 0:56	2:54 ± 0:45	0.721
Cold ischemic time, h:m	21:56 ± 0:37	22:40 ± 0:36	22:41 ± 0:22	20:51 ± 0:57	0.120
Anastomotic time, min	30 ± 5	32 ± 4	28 ± 3	26 ± 3	0.203
Total ischemic time, h:m	22:56 ± 0:35	23:42 ± 0:35	23:20 ± 0:24	23:09 ± 0:29	0.170

HMP, hypothermic machine perfusion; HMPO_{high}, high oxygenated hypothermic machine perfusion; HMPO_{low}, low oxygenated hypothermic machine perfusion; SCS, static cold storage.

to animal and organ size. Serum creatinine was analyzed using a longitudinal mixed model for repeated measurements (MMRM) with main factors treatment, timepoint, and interaction term to test for overall difference in graft function over time between treatment groups. Individual comparison of groups was conducted using contrast analyses within the main MMRM model testing the null hypothesis that the difference in least squares means between 2 groups under investigation was zero (no mean difference between groups). For the contrast analysis, *P* values (based on *t* test of least squares means) were reported along with a graphical mean (SE) plot where deemed appropriate. All secondary endpoint variables were similarly modeled to the primary endpoint. In case of variables without longitudinal structure (eg, area under the curve [AUC]), the MMRM model was reduced to a basis ANOVA model with factor treatment. As this was a preclinical exploratory trial, no formal sample size calculations were performed and no correction for multiplicity was foreseen. All analyses were performed using SAS9.2 or SAS 9.4 (SAS Institute, Cary, NC) and Prism 8.2.0 (Graphpad Software, San Diego, CA).

RESULTS

Operative Data, Major Complications, and Animal Survival

Thirty-two autotransplants were performed. Four were excluded from the analysis for death in relation to complications (sudden death [*n* = 1], bleeding central line damage [*n* = 1], necrotizing anal prolapse [*n* = 1], massive bleeding after reperfusion [*n* = 1]). The operative data of the 28 animals are reported in Table 1.

Graft Function

The AUC analysis of the serum creatinine from day 1 until 13 after transplantation is shown in Figure 2A. Comparison of the study groups based on the main MMRM demonstrated a significant difference in favor of both HMPO₂ groups compared with SCS (*P* < 0.0001) and standard HMP

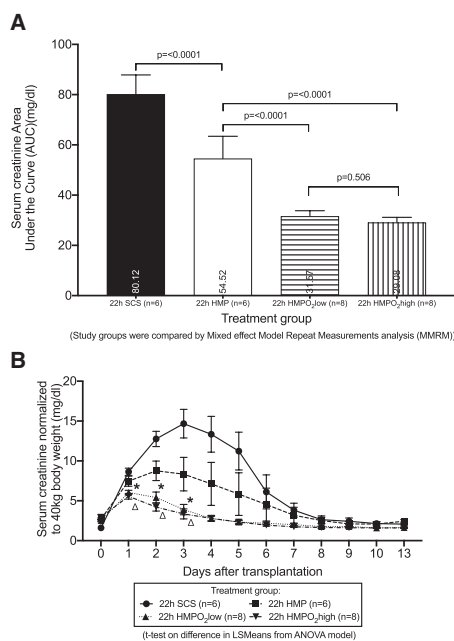


FIGURE 2. Area under the curve analysis of serum creatinine from d 1 until d 13 after transplantation according to the study group demonstrating that both continuous active oxygenated study groups significantly improved early graft function compared with SCS alone and passive aerated HMP (A). Evolution of daily postoperative serum creatinine in all study groups (B). *Significance of *P* < 0.05 between HMP and HMPO_{low} group. ^Significance of *P* < 0.05 between HMP and HMPO_{high} group. AUC, area under the curve; HMP, hypothermic machine perfusion; HMPO_{low}, oxygenated hypothermic machine perfusion; LS, least square; SCS, static cold storage.

($P < 0.0001$). No difference could be demonstrated between both HMPO₂ groups ($P = 0.506$). The evolution of serum creatinine after transplantation is shown in Figure 2B. No statistically significant difference was observed between the HMPO₂high and low oxygenated HMP (HMPO₂low) groups during the follow-up period. During the first 3 days after autotransplantation, the serum creatinine in these 2 groups was significantly lower ($P < 0.05$) compared with standard HMP and SCS alone.

Tubular reabsorption of sodium was transiently disturbed in all study groups after transplantation but demonstrated a faster decrease to baseline values during the first 3 postoperative days in all machine perfusion strategies compared with the SCS group (Figure 3A). The AUC analysis of the fractional excretion of sodium demonstrated a significant difference between the SCS group and the HMPO₂high group (Figure 3B). The same findings were observed for the urinary protein creatinine ratio (Figure 3C and D).

Evolution of Postoperative Serum LDH and AST

In all study groups, the peak of serum LDH was observed at day 1 after transplantation and dropped immediately afterward, normalizing after 6 days. No significant difference was observed in the postoperative LDH profile after autotransplantation between all the study groups

(Figure 4A). The same findings were seen in relation to the postoperative AST profile (Figure 4B).

Renal Flow and Resistance During Machine Perfusion

Renal flow during machine perfusion was significantly higher in both HMPO₂ groups compared with standard HMP during the entire period of machine perfusion (Figure 5A). No significant difference in renal flow was observed between both HMPO₂ groups during the whole period of machine perfusion. The same findings were seen for renal resistance (Figure 5B).

Perfusate Po₂ Levels

High Po₂ resulted in a 3- and 10-fold increase in perfusate Po₂ compared with HMPO₂low and standard HMP, respectively (Table 1). The perfusate Po₂ at the start and the end of machine perfusion was 170 ± 4 and 70 ± 5 mmHg, 217 ± 9 and 222 ± 8 mmHg, and 726 ± 41 and 718 ± 40 mmHg, respectively, for the standard HMP, the HMPO₂low, and HMPO₂high group measured at 37°C.

Perfusate Glucose, LDH, and AST

No difference was found in perfusate glucose between the different machine perfusion groups. LDH was

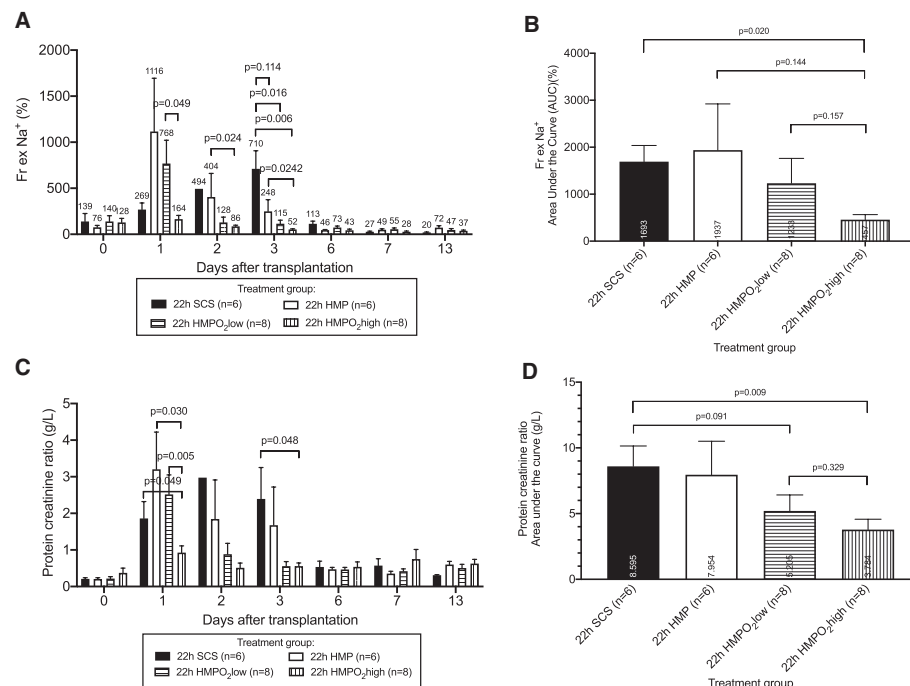


FIGURE 3. The daily evolution and the area under the curve analysis of the urinary fractional excretion of sodium and protein creatinine ratio according to the study groups (A and B and C and D, respectively). AUC, area under the curve; Fr ex Na⁺, fractional excretion of sodium; HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion; SCS, static cold storage.

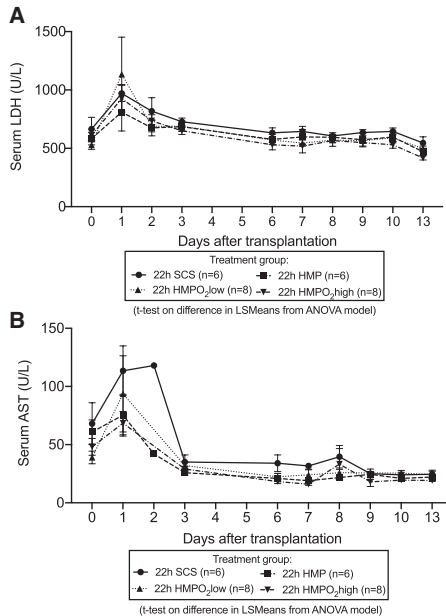


FIGURE 4. Evolution of post-transplant serum LDH and AST in the continuous hypothermic machine perfusion groups with or without active oxygenation and the static cold storage group (A and B, respectively). AST, aspartate transaminase; HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion; LDH, lactate dehydrogenase; LS, least square; SCS, static cold storage.

comparable between the HMP group and the HMPO₂low group, however was significantly lower at the end of machine perfusion in the HMPO₂high group compared with the HMPO₂low group ($P = 0.018$). Perfusate AST was significantly higher at the end of machine perfusion in the HMPO₂low group compared with the HMPO₂high group ($P = 0.0007$) and HMP group ($P = 0.0007$) (Figure 6).

¹H-NMR: Quantification of Perfusate Metabolites Concentrations

¹H-NMR analysis was used to determine the concentration of metabolites within the circulating perfusate at the end of perfusion (Table S1, SDC, <http://links.lww.com/TP/B842>). A significantly lower concentration of major central metabolites lactate and formate was observed in the HMPO₂high group compared with the standard HMP and the HMPO₂low groups. Significantly reduced concentrations of succinate and acetate were observed in both active oxygenated groups compared with the standard HMP group (Figure 7).

Oxidative Stress and Metabolic Evaluation on Preimplantation Biopsies

HIF-1 α expression on preimplantation biopsies by Western blotting was significantly lower in both HMPO₂

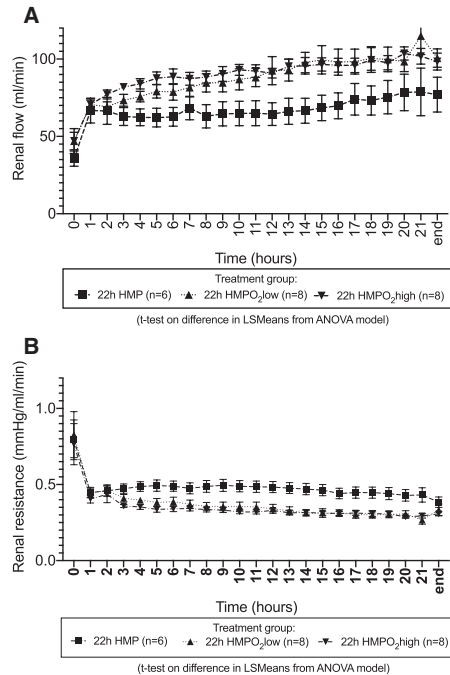


FIGURE 5. Influence of oxygen supply on ex vivo renal flow (A) and renal resistance (B) during continuous hypothermic machine perfusion with or without active oxygenation. HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion; LS, least square.

groups compared with the SCS group (Figure 8). NOX-2 expression was significantly lower in the standard HMP and HMPO₂low group compared with standard SCS ($P = 0.016$ and $P = 0.034$, respectively). No significant difference in NOX4, HO-1, SOD1, and peroxiredoxin 1 protein expressions have been observed between all study groups.

Metabolic quantification by liquid chromatography electrospray ionization mass spectrometry demonstrated that central metabolites succinate and glutamate at the end of the preservation were significantly lower in both HMPO₂ groups compared with the SCS and standard HMP groups (Figure 9A and B). No differences were observed in lactate, ATP, ADP, AMP, and NADH and oxidized nicotinamide adenine dinucleotide concentrations between all study groups (Figure 9C–F) (data not shown for NADH and oxidized nicotinamide adenine dinucleotide). Although no significant difference between the groups in terms of ATP production was observed at the end of the preservation, ATP was very low in the SCS group compared with all HMP groups.

Histology

Acute tubular injury assessed by the Banff score was significantly lower on the preimplantation biopsies in favor of the SCS group compared with all machine perfusion

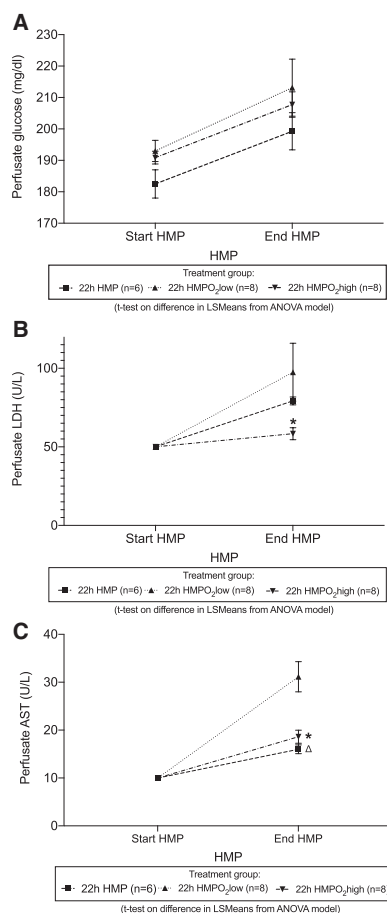


FIGURE 6. Evolution of perfusate glucose, LDH, and AST during continuous hypothermic machine perfusion with and without active oxygenation (A, B, C, respectively). *Significance of $P < 0.05$ between HMPO₂low and HMPO₂high group. ^Significance of $P < 0.05$ between the HMPO₂low and the HMP group. AST, aspartate transaminase; HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion; LDH, lactate dehydrogenase; LS, least square.

groups independent of oxygen supply ($P < 0.0001$); nevertheless, no differences were observed between all machine perfusion groups. Assessment of the same preimplantation biopsies by the Hosgood score demonstrated only a significantly lower score in favor of the SCS groups compared with the HMP and HMPO₂high groups ($P = <0.0001$ and $P = 0.050$, respectively). No difference was observed between the SCS and HMPO₂low group and also no differences were observed between all machine perfusion groups. No differences were observed between all groups

for all other Banff criteria (eg, interstitial fibrosis, tubular atrophy, interstitial inflammation, arterial intimal fibrosis, arteriolar hyalinosis, and glomerular thrombi). No significant differences were observed between all groups in the baseline and the end biopsies (data not shown).

DISCUSSION

This preclinical animal study adds to the body of evidence evaluating the role of oxygenation during HMP. Active perfusate oxygenation (HMPO₂low and HMPO₂high) resulted in improved early graft function and ex vivo renal flow during machine perfusion compared with SCS alone and standard HMP. Lactate and succinate perfusate analysis by ¹H-NMR analysis demonstrated significantly lower concentrations in favor of the HMPO₂high group at the end of machine perfusion. Preimplantation biopsies demonstrated a significant reduction of central metabolites succinate and glutamate in both HMPO₂ groups compared with standard HMP and SCS alone. No difference could be observed in preimplantation ATP levels between all groups. We found, as expected, that the Po₂ within the acellular perfusion fluid (with no designated oxygen carrier) can be dramatically increased during active oxygenation and is dependent on the percentage of oxygen concentration delivered during HMP. In contrast, during standard HMP, the perfusion fluid oxygen was rapidly diminished, resulting in a relatively hypoxic perfusion environment at the end of the machine perfusion period thus hindering the oxygen-dependent processes that occur during HMP. However, no significant difference in pro- or antioxidants expression (NOX4, HO-1, SOD-1, peroxiredoxin 1) was observed between all groups on preimplantation biopsies. In contrast, HIF-1 α expression was significantly lower in both HMPO₂ groups compared with SCS alone. No important structural differences on the preimplantation and the end biopsies were observed by light microscopy between all machine perfusion groups.

An overview of the literature of preclinical porcine kidney studies investigating different oxygen concentrations during continuous HMP and their effect on early graft function and ex vivo renal flow, using ex vivo normothermic reperfusion or an autotransplant model is summarized in Table 2 and shows conflicting results.^{4,5,7,16} Interestingly, in autotransplant models, the functional benefits of oxygen seem to correlate with the ischemic insult, with comparable functional benefits reported following 30 or 60 minutes of preceding warm ischemia before preservation. In the absence of preceding warm ischemia, no benefit of active oxygenation was observed.⁷ Despite the improved flow and function demonstrated by oxygenation in this study, we found no difference in functional outcome measures between the low and high perfusate Po₂ conditions.

Chouchani et al^{17,18} demonstrated that mitochondrial succinate accumulation (due to reversed electron transport) originated from the citric acid cycle during ischemia is responsible for the major part of the reactive oxygen species production during ischemia-reperfusion, independent of the type of organ in the human body. In addition, Dutkowski et al¹⁹ recently described that end-ischemic oxygenated hypothermic liver perfusion results in forward electron transport at the level of the mitochondrial respiratory chain during HMP. This results in a decrease in succinate and the increase

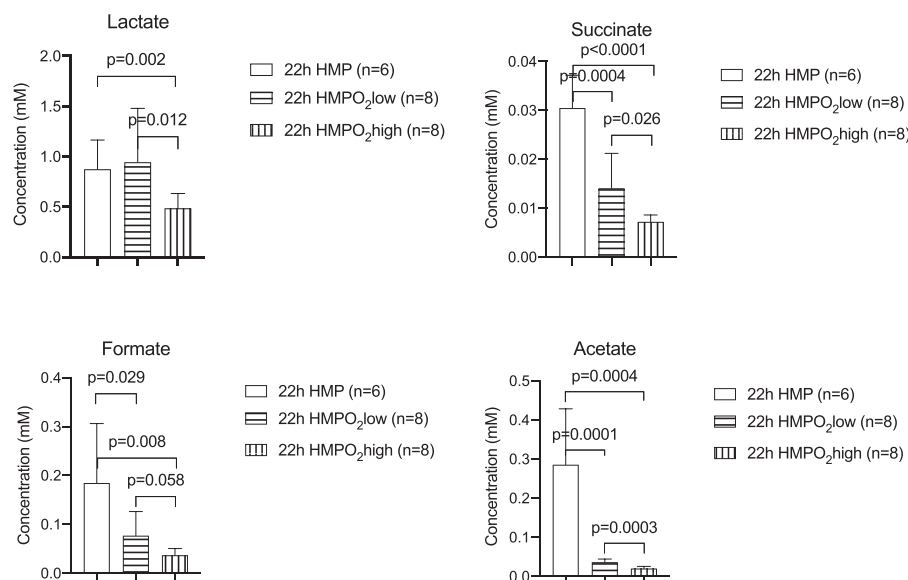


FIGURE 7. Concentrations of lactate, succinate, formate, and acetate in circulating perfusion fluid at the end of machine perfusion in all machine perfusion groups. HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion.

of ATP at the end of HMP and subsequent improved graft function. Our study results are in line with these observations demonstrating a significant decrease of central metabolites succinate and glutamate on preimplantation biopsies and an improved initial graft function in the presence of supraphysiological perfusate Po₂ compared with standard HMP or SCS alone. The SCS alone group demonstrated the lowest ATP level at the end of the preservation compared with all HMP groups. The high variability of ATP measurements was probably the main reason that our study was not able to detect a significant difference between all study groups and could have been introduced during the samples acquisition, storage, or extraction process.

While this study does not demonstrate functional differences between the 2 oxygenated conditions, there are clear metabolic consequences of supraphysiological perfusate oxygenation. We found that the perfusate concentration of major central metabolites like lactate, succinate, formate, and acetate at the end of machine perfusion inversely correlated with the oxygen concentration added during machine perfusion. Such a phenomenon cannot be explained by the washout of metabolites from the kidney, as this would cause the converse (higher concentrations in high-flow oxygenated conditions). One possible explanation could be that in oxygen abundant conditions, the efficiency of cellular metabolism improves and therefore overall anaerobic metabolic activity is diminished. This is supported by previous studies demonstrating that while the oxygen-dependent Krebs cycle is upregulated in oxygen-rich environments, other pathways become less active resulting in an overall metabolic “quiescence.”⁸ This relationship appears to be oxygen dose

dependent with maximal amount of Krebs cycle activity and corresponding increase in cellular ATP levels occurring with high flow oxygen. Interestingly, while Krebs-related activity increases, the absolute concentration of the Krebs intermediates themselves, such as succinate and citrate were diminished in the high oxygen groups, highlighting the limitations of isolated metabolite quantification. We are aware that the origin of perfusate succinate is multifactorial, with higher perfusate concentrations indicating any combination of upregulated manufacture, down-regulated utilization, increased export, or increased release under necrosis.

Cell membrane damage may be inferred by increased LDH level detected in the perfusate.^{20,21} Higher levels of LDH may coincide with increased leakage of cellular metabolites into the perfusate. We observed higher levels of perfusate LDH in the standard HMP and HMPO₂low groups when compared with the HMPO₂high group. Therefore, improvements in cell membrane integrity under HMPO₂high may help to explain the reductions in perfusate lactate and succinate seen in this group. To determine the significance of perfusate metabolites, advanced analytical tools are required. Tracer studies using isotopically labeled metabolic substrates are a useful tool, which can elucidate whether a detected metabolite has been produced de novo. Using a combination of NMR and mass spectrometry and an ex vivo donation after cardiocirculatory death porcine model of HMP, Patel et al⁸ recently demonstrated that perfusate supplementation with high concentration of oxygen (95%) results in a greater degree of aerobic metabolism at the end of machine perfusion versus active aeration (21%). In contrast, we did not add the

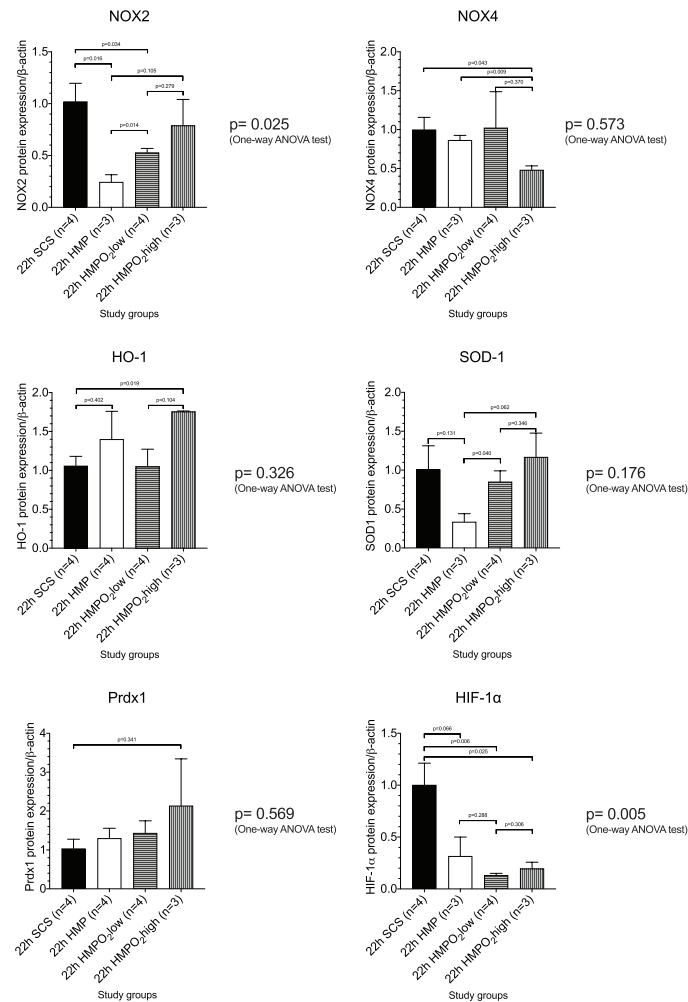


FIGURE 8. HIF-1 α expression on preimplantation biopsies by Western blotting was significantly lower in both oxygenated HMP groups compared with the SCS group. NOX-2 expression was significantly lower in the standard HMP and HMPO₂low group compared with standard SCS. No significant difference in NOX4, HO-1, SOD1, and Prdx1 protein expressions have been observed between all study groups. HIF-1 α , hypoxia-inducible factor 1 α ; HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion; HO-1, heme oxygenase 1; NOX4, nicotinamide adenine dinucleotide phosphate oxidase 4; Prdx1, peroxiredoxin 1; SCS, static cold storage; SOD1, superoxide dismutase 1.

universally labeled glucose ([U-¹³C] glucose) to the perfusate fluid in our study protocol. However, in line with their results, we observed in our study that the perfusate lactate concentration by ¹H-NMR analysis was significantly lower at the end of machine perfusion in the highly oxygenated compared with the low oxygenated and the non-active HMPO₂ group. Lactate is considered as a universal marker of anaerobic metabolism. Therefore, this result can

be interpreted that supraphysiological levels of oxygen in the perfusate more efficiently promote a metabolic switch to aerobic metabolism during cold machine perfusion compared with low perfusate oxygen levels.

HIFs-1 α are the universal cellular oxygen-sensitive transcription factors that activate a number of hypoxia-responsive genes, including vascular endothelial growth factor, erythropoietin, epidermal growth factor receptor, and

platelet-derived growth factor.²² HIF-1 α normally accumulates during ischemia but was significantly decreased in this study in the presence of active oxygenation during HMP.²³ Therefore, HIF-1 α expression could play a major role in early and late allograft function after HMPO₂, as also previously described.²² NOX2 is involved in the pathogenesis of ischemia-reperfusion injury.²⁴ NOX2 protein expression is weakly expressed in standard HMP and HMPO₂low suggesting that oxidative stress is lower in these groups. NOX2 activation requires subunits interaction (mainly, p47-phox and p22-phox) to rule on its activation state.

Histological evaluation of the preimplantation biopsies using the Banff score¹⁴ and the Hosgood score¹⁵ focusing on the degree of tubular injury did not correlate with initial graft function in our study groups. This could reflect a limitation of our autotransplant model using young, healthy pigs without preexisting kidney disease and only 30 minutes of preceding warm ischemia time before procurement. However, 30 minutes of warm ischemia is similar to the mean first warm ischemia time in most clinical controlled donation after cardiocirculatory death kidney transplant programs and is frequently used in preclinical models.^{2,4,16} Tubular reabsorption of sodium was transiently disturbed

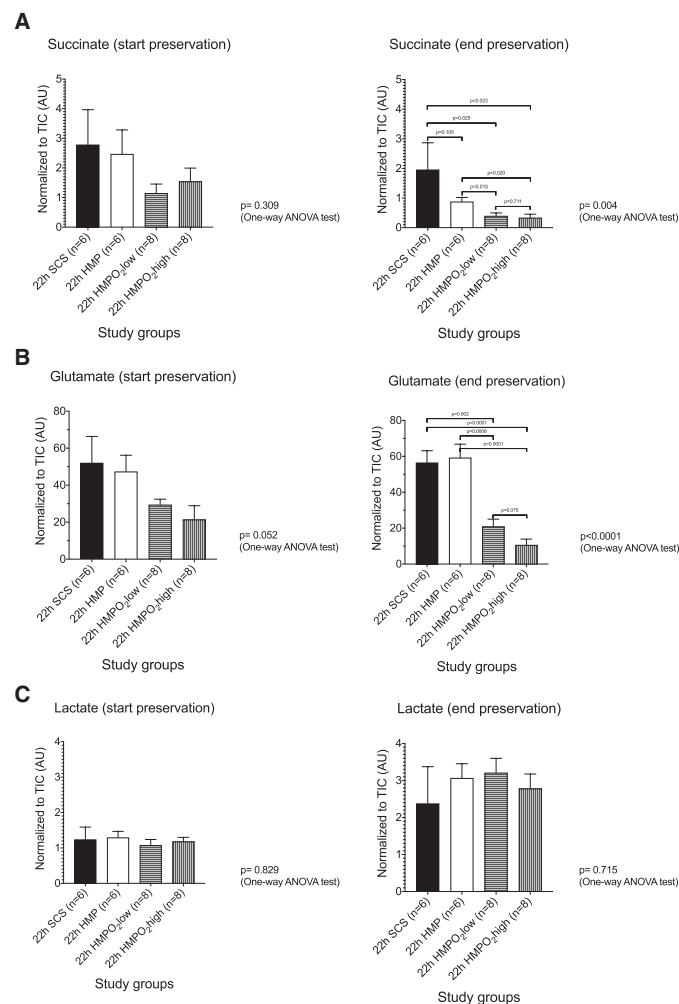


FIGURE 9. Continue

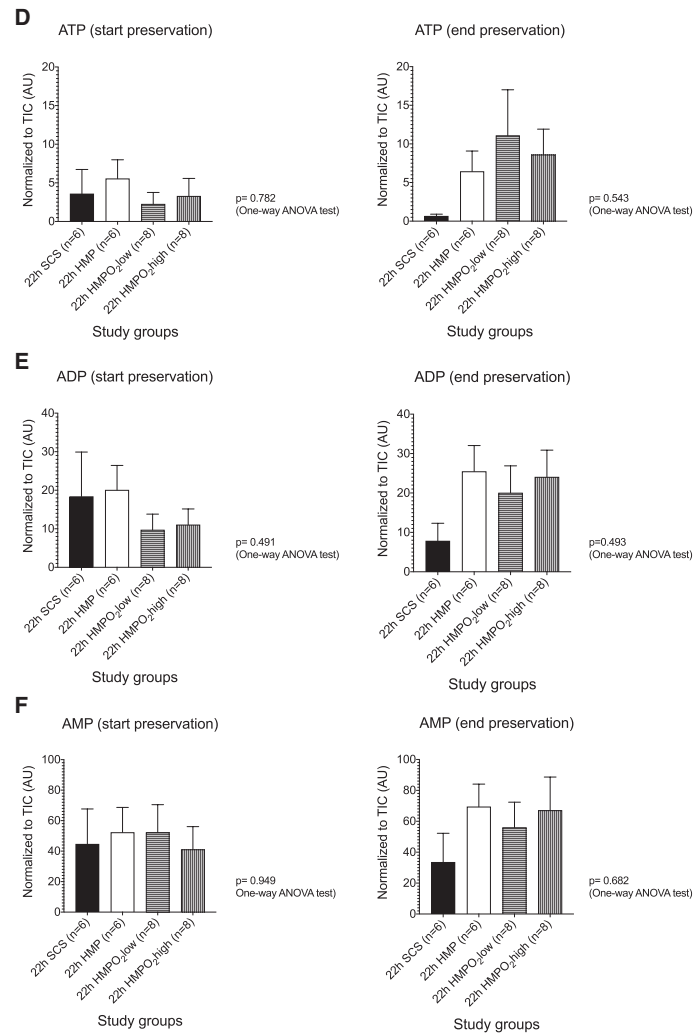


FIGURE 9. Metabolic quantification by LC-ESI-MS demonstrated that central metabolites succinate (A) and glutamate (B) at the end of the preservation were significantly lower in both oxygenated HMP groups compared with the SCS and standard HMP groups. No differences were observed of lactate (C), ATP (D), ADP (E), and AMP (F) in all study groups. Although no significant difference between the groups in terms of ATP production was observed at the end of the preservation, ATP was very low in the SCS group compared with all HMP groups. AU, arbitrary unit; HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion; LC-ESI-MS, liquid chromatography electrospray ionization mass spectrometry; SCS, static cold storage; TIC, total ion current.

in all study groups but demonstrated a faster decrease to baseline especially during the first 3 days after transplantation in all machine perfusion strategies, independent of oxygen supply compared with SCS alone. In contrast, acute tubular injury assessed by the Banff score¹⁴ was even significantly lower on the preimplantation biopsies in

favor of the SCS group compared with all machine perfusion groups independent of oxygen supply.

In conclusion, this ischemia-reperfusion porcine autotransplant model demonstrated a significant functional benefit from active supraphysiological oxygenation during HMP on initial graft. Perfusate metabolic profile

TABLE 2.
Overview of preclinical pig studies investigating different oxygen concentrations during continuous hypothermic machine preservation of kidneys using an ex vivo normo-thermic reperfusion or an autotransplant model

Author, year of publication	Model	Study groups (number per group)	pO ₂ , mmHg at 37 °C	Initial graft function (clearance ^a , serum creatinine ^b)	Evolution of renal flow during HMP	Renal flow at the end
Ex vivo normothermic reperfusion model Hoyer et al, 2014 ⁴	30 min WI + 21 h preservation	1. 21 h HMP 0% O ₂ (n = 5)	0	⁴ HMP 100% O ₂ provides the best initial graft function, followed by HMP 20% O ₂ and HMP 0% O ₂	Fastest increase of renal flow using HMP 100% O ₂ , followed by HMP 20% O ₂ and HMP 0% O ₂	HMP 100% O ₂ and HMP 20% O ₂ significantly higher than HMP 0% O ₂
		2. 21 h HMP air (n = 5)	200–220			
		3. 21 h HMP 100% O ₂ (n = 5)	760–800			
Autotransplant model Venema et al, 2018 ¹⁶	30 min WI + 24 h preservation	1. 24 h SCS (n = 6)	–	⁴ All HMP groups significantly better than SCS group	NA	NA
		2. 24 h HMP 0% O ₂ (n = 6)	0			
		3. 24 h HMP 21% O ₂ (n = 6)	NA			
		4. 24 h HMP 100% O ₂ (n = 6)	NA			
Autotransplant model Gallinat et al, 2012 ⁷ Thuillier et al, 2013 ⁵	No WI + 21 h preservation 60 min WI + 22 h preservation	1. 21 h HMP 0% O ₂ (n = 5)	0	⁴ Significantly better after HMP 0% O ₂ compared with HMP 100% O ₂	NA	NA
		2. 21 h HMP 100% O ₂ (n = 5)	>500			
		1. 22 h HMP 0% O ₂ (n = 4)	0			
		2. 22 h HMP 100% O ₂ (n = 4)	NA			
Autotransplant model Darius et al, 2019 ²	30 min WI + 22 h preservation	1. 22 h SCS (n = 6)	–	⁴ HMP 0% low and HMP 0% high demonstrated significant better initial graft function compared with standard HMP	Faster increase in both HMP 0% high and HMP 0% low groups compared with standard HMP but no significant difference between HMP 0% high and HMP 0% low	Significantly higher in both HMP 0% high and HMP 0% low compared with standard HMP
		2. 22 h standard HMP (n = 6)	65–75			
		3. 22 h HMP 0% low (n = 8)	210–230			
		4. 22 h HMP 0% high (n = 8)	680–760			

^aMeasured by clearance.

^bMeasured by serum creatinine.

HMP, hypothermic machine perfusion; HMP 0% low, oxygenated hypothermic machine perfusion; NA, not available; pO₂, partial pressure of oxygen; SCS, static cold storage; WI, warm ischemia.

analysis suggests that aerobic mechanism is better supported under high perfusate Po_2 conditions compared with standard HMP and HMPO_2 low but no difference between high and low perfusate Po_2 was observed by oxidative stress and metabolic evaluation on preimplantation biopsies. The next question to be answered is to determine the optimal start time of active supraphysiological oxygen administration during continuous HMP.

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Summary Paper 2

Aim of the study:

The aims of the study were to determine the functional, metabolic, structural, and flow dynamic effects of low and high perfusion fluid oxygen concentration during hypothermic machine perfusion (HMP) in a porcine ischemia-reperfusion DCD autotransplant model.

Main conclusions:

- No difference in functional outcome was observed between the low (30% Carbogen (95% O₂ / 5% CO₂)) and the high oxygenated (90% Carbogen (95% O₂ / 5% CO₂)) HMP groups, but both groups were superior to standard HMP without active oxygenation.
- Active oxygenation during HMP results in a higher renal flow which leads to an increased oxygen delivery to the tissues.
- High oxygen concentration (90% Carbogen) during HMP might be the best preservation strategy to drive the metabolic switch to aerobic metabolism.
 - Lactate level (= marker for anaerobic metabolism) is lower during HMP when high oxygen concentration is used as compared with low dose of oxygen during machine perfusion.
 - ATP (Adenosine triphosphate) generation during HMP was higher when oxygen added during perfusion.
 - Succinate (responsible for mitochondrial reactive oxygen species production during in vivo reperfusion) concentration was the lowest in the high oxygenated HMP group (in tissue and perfusion fluid).
 - Hypoxia inducible factor 1 alpha (impacts negatively early graft function) was significantly lower in all machine perfusion groups with or without additional oxygen supply.

Personal contributions
Results: Paper 3

Paper 3: Brief O₂ uploading during continuous hypothermic machine perfusion is simple yet effective oxygenation method to improve initial kidney function in a porcine autotransplant model.

Personal contributions
Results: Paper 3



Brief O₂ uploading during continuous hypothermic machine perfusion is simple yet effective oxygenation method to improve initial kidney function in a porcine autotransplant model

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With oxygenation proposed as a resuscitative measure during hypothermic models of preservation, the aim of this study was to evaluate the optimal start time of oxygenation during continuous hypothermic machine perfusion (HMP). In this porcine ischemia-reperfusion autotransplant model, the left kidney of a ± 40 kg pig was exposed to 30 minutes of warm ischemia prior to 22 hours of HMP and autotransplantation. Kidneys were randomized to receive 2 hours of oxygenation during HMP either at the start ($n = 6$), or end of the perfusion ($n = 5$) and outcomes were compared to standard, nonoxygenated HMP ($n = 6$) and continuous oxygenated HMP ($n = 8$). The brief initial and continuous oxygenated HMP groups were associated with superior graft recovery compared to either standard, nonoxygenated HMP or kidneys oxygenated at the end of HMP. This correlated with significant metabolic differences in perfusate (eg, lactate, succinate, flavin

Abbreviations: ¹H-NMR, proton nuclear magnetic resonance; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ANOVA, analysis of variance; AST, aspartate aminase; ATP, adenosine triphosphate; AUC, area under the curve; CAC, citric acid cycle; FET, forward electron transport; FMN, flavin mononucleotide; Fr ex Na, fractional excretion of sodium; HIF-1 α , hypoxia-inducible factor 1 α ; HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion; HO-1, heme oxygenase 1; KPS-1, kidney perfusion solution-1; LC-ESI-MS, liquid chromatography electrospray ionization mass spectrometry; LDH, lactate dehydrogenase; MMRM, mixed model for repeated measures; NAD⁺, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; Nr12, nuclear factor erythroid 2-related factor 2; pO₂, partial pressure of oxygen; RET, reverse electron transfer; ROS, reactive oxygen species; SD, standard deviation; SOD1, superoxide dismutase 1.

mononucleotide) and tissues (eg, succinate, adenosine triphosphate, hypoxia-inducible factor-1 α , nuclear factor erythroid 2-related factor 2) suggesting superior mitochondrial preservation with initial oxygenation. Brief initial O₂ uploading during HMP at procurement site might be an easy and effective preservation strategy to maintain aerobic metabolism, protect mitochondria, and achieve an improved early renal graft function compared with standard HMP or oxygen supply shortly at the end of HMP preservation.

KEYWORDS

animal models; porcine, autotransplantation, ischemia-reperfusion injury (IRI), kidney (allograft) function/dysfunction, kidney transplantation/nephrology, organ perfusion and preservation, organ procurement and allocation, translational research/science

1 | INTRODUCTION

Several preclinical studies have shown a protective effect of supplemental oxygenation (continuous or end preservation) during hypothermic machine perfusion (HMP).¹⁻⁵ However, the optimal protocol to deliver oxygen during cold perfusion remains unclear. Our group compared different concentrations of partial oxygen pressure (pO₂) in the perfusate during continuous HMP in a porcine ischemia-reperfusion autotransplant model.^{6,7} Although no differences in functional outcomes were observed between the low (30%) and the high (90%) perfusate pO₂ groups, aerobic mechanisms were better supported under high oxygen concentrations,⁷ confirming previous preclinical studies.^{2,8,9} Such preimplant metabolic optimization could help to abrogate the harmful effects of ischemia-reperfusion injury (IRI), with particular benefit in an era of frequent high-risk donor organ utilization, with associated morbidity.¹⁰

Furthermore, it remains unclear if additional oxygen is required during the entire preservation period. Reducing the duration of oxygen delivery to a short period either following organ retrieval or prior to implantation could confer significant logistical benefits. This may be particularly important where airplane travel is required for organ.

We therefore evaluate here the impact of start time and duration of active oxygenation during continuous HMP on early graft function in a pig kidney ischemia-reperfusion autotransplant model. We further measure renal flow, resistance and perfusate pO₂ during machine perfusion, and assess tubular function. Biomarkers and metabolic profiles are obtained from perfusate and renal biopsies.

2 | MATERIALS AND METHODS

2.1 | Animals

Female Belgian Landrace pigs of approximately 40 kg were used. Experiments were performed according to international guidelines, after approval by the local ethical committee for animal care. The

animals were housed and cared for according to Belgian laws on experimental animal welfare.

2.2 | Study design

A standardized ischemia-reperfusion porcine kidney autotransplant model was used as previously described.⁶

2.3 | Anesthetic and surgical protocol

Anesthetic and surgical protocols were followed as described previously.^{6,7} The left kidney was removed after 30 minutes of warm ischemia induced by vascular clamping of the renal vessels and subsequently flushed with 200 mL of Kidney Perfusion Solution (KPS-1, Organ Recovery Systems, Diegem, Belgium). The kidney was randomized to one of the following preservation strategies: (1) 2-hour oxygenated HMP (90% oxygen) (HMPO₂) + 20-hour HMP with no additional oxygen (n = 6) or (2) 20-hour HMP followed by 2-hour HMPO₂ (n = 5). Historical control groups were 22-hour standard HMP (n = 6) with no additional oxygen in the perfusate and 22-hour continuous oxygenated HMP (HMPO₂) (n = 8) (90% oxygen). Following preservation, the left kidney was autotransplanted after a right nephrectomy.⁶

The surviving animals were euthanized on day 13 after transplantation by an intravenous injection of T61® (Intervet International, MSD Animal Health, Madison, NJ) under general anesthesia.

2.4 | Machine perfusion

The LifePort Kidney Transporter® (Organ Recovery Systems) was used for all machine perfusion groups. In the active oxygenated study groups, the circuit was modified to include an oxygenator (Dideco Kids D100 neonatal oxygenator, Sorin Group, Saluggia, Italy) and

heat exchanger as described previously.^{6,7} The KPS-1 solution was pumped through the kidney at a pressure of 30 mm Hg. Carbogen (95% O₂/5% CO₂) and not 100% oxygen was used for oxygen supplementation at 90% in the experimental groups to have identical conditions as compared with the historical oxygenated group. The temperature of the kidney and the circulating perfusate was maintained at 2–8°C.

2.5 | Outcome measures

The primary outcome measure was early graft function (measured as daily serum creatinine). The secondary outcome measures were tubular function (measured by fractional excretion of sodium [Fr ex Na] and protein creatinine ratio in the urine), serum biomarkers (lactate dehydrogenase [LDH] and aspartate transaminase [AST]), renal flow, resistance and pO₂ during machine perfusion, metabolic perfusate and tissue profile and graft histology of the corresponding preimplantation kidney graft biopsy.

2.6 | Samples and analyses

2.6.1 | Renal flow and resistance measurement during HMP and perfusate analyses

The machine perfusion device continuously registers renal flow and resistance. Perfusate samples were taken at start, 15, 30, 60, 120 minutes, and at the end of machine perfusion. Perfusate pO₂ was continuously measured during perfusion by a micro fiber oxygen transmitter (Oxy-4 micro, Precision Sensing GmbH, Regensburg, Germany). Glucose, AST, and LDH analyses were performed by a dry chemistry analyzer Fui Dri-Chem NX500 (Fujifilm Corporation, Tokyo, Japan). For metabolite identification and quantification using nuclear magnetic resonance (NMR) spectroscopy, neat perfusate samples were mixed with a concentrated NMR buffer, granting end concentrations of 0.5 mmol/L DSS, 0.1 mol/L phosphate buffer, 25% D₂O and 2 mmol/L imidazole for each sample. Proton nuclear magnetic resonance (¹H-NMR) spectra were acquired on a 600-MHz Bruker Avance III NMR spectrometer in 5 mm NMR tubes. The spectra were phase and baseline corrected with Metabolab software.¹¹ Metabolite identification and quantification was performed using Chenomx NMR Suite (Chenomx, Edmonton, Alberta, Canada). Metabolic profiles were assessed at the perfusion start, after 2 hours and at the end. The following metabolites were quantified: acetate, adenine, alanine, aspartate, formate, gluconate, glutamate, glutathione, glycine, hypoxanthine, isoleucine, lactate, leucine, mannitol, myo-inositol, and succinate. Perfusate samples were also analyzed with the spectrometer (fluorescence filter at 525 nm) to detect the amount of flavin mononucleotide (FMN) release at the beginning of perfusion, after 15, 30, 60, 120 minutes, and at the end of HMP.

2.6.2 | Blood and urine analysis

Venous blood samples were taken daily during follow-up. Urine samples were collected for biochemical analysis. Blood and urine analyses were determined using routine clinical methods.

2.6.3 | Tissue sampling and analysis

Kidney biopsies were taken at five different time points. Wedge biopsies were taken before the onset of preservation after ex vivo flush with KPS-1, after 22 hours of preservation, and before euthanasia. Needle biopsies of the kidney graft were taken at 5 and 45 minutes after in vivo reperfusion. Biopsies were stored both, fresh at –80°C pending metabolic analysis and also fixed in acidified formal alcohol, dehydrated, and embedded in paraffin wax for histology. Protein expression levels were determined by western blot analysis using start and end preservation and reperfusion samples of frozen kidney biopsies as previously described.¹² Levels of oxidative enzymes such as heme oxygenase 1 (HO-1) (Abcam ab13243), antioxidant enzymes such as superoxide dismutase 1 (SOD1) (Abcam ab13498), and Nuclear factor erythroid 2-related factor 2 (Nrf2) (Abcam ab92946) and reactive oxygen species (ROS) sensitive protein such as hypoxia-inducible factor 1α (HIF-1α) (BD transduction, BD 610959), have been detected and were normalized to the levels of β-actin. Metabolites (lactate, succinate, glutamate, adenosine monophosphate [AMP], adenosine diphosphate [ADP], adenosine triphosphate [ATP] and oxidized and reduced nicotinamide adenine dinucleotide [NAD⁺ and NADH, respectively]) were extracted from frozen kidney biopsies by methanol-chloroform extraction and analyzed by liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS) using a method based on Coulier et al.¹³ Metabolite quantification was performed as described by Veiga-da-Cunha et al.¹⁴

Sections of 4 μm were cut and stained with hematoxylin and eosin for evaluation using light microscopy. Sections were scored blindly assessing changes according to the histopathological consensus criteria for preimplantation kidney biopsies¹⁵ and the acute tubular injury score described by Hosgood et al.¹⁶ to assess changes in four morphological categories: tubular dilatation, tubular debris, vacuolation, and infiltration.

2.7 | Statistical methods

The study follows a parallel design with four active groups. No imputation was done, so all analyses were based on the observed cases only.

The primary outcome measure of early graft function was assessed through analysis of serum creatinine over time using generalized linear model techniques. Serum creatinine values were normalized to 40 kg animal body weight prior to use in the analysis to correct for differences in animal and organ size. Serum creatinine was analyzed using mixed model for repeated measurements

(MMRM) with factors treatment, time-point, and interaction term to test for overall difference in graft function over time between groups. Individual comparison of groups were conducted using contrast analyses within the main MMRM model testing the null hypothesis that the least squares mean difference between two groups was zero (no mean difference). For the contrast analysis, *P* values (based on *t* test of least squares means) were reported along with a mean (SD) graph where appropriate. All secondary outcome variables were similarly modeled to the primary outcome measure. In case of variables without repeated measurement (eg, area under the curve [AUC]) the MMRM model was reduced to an analysis of variance (ANOVA) model with factor treatment. As this was a preclinical exploratory trial, no formal sample size calculations were performed and no correction for multiplicity was foreseen. All analyses were performed using SAS 9.4 (SAS Institute, Cary, NC) and Prism 8.2.0 (GraphPad Software, LaJolla, CA).

3 | RESULTS

3.1 | Operative data, major complications, and animal survival

Twenty-nine autotransplants were performed and four were excluded from the analysis due to early death or graft-interfering complications (sudden death at day 2 [*n* = 1], hydronephrosis of the right kidney diagnosed during the autotransplant procedure because of mechanical

obstruction caused by the balloon of the bladder catheter [*n* = 1], respiratory arrest 30 minutes after extubation on day 0 [*n* = 1], necrotizing anal prolapse at day 5 necessitating euthanasia [*n* = 1]). Operative data of the remaining 25 autotransplantations are presented in Table 1. The number of pigs in the nonhistorical study groups was reduced to 11 during the project, based on an intermediate statistical analysis, instead of the foreseen 16 to limit animal suffering.

3.2 | Graft function

The AUC of the serum creatinine within 13 days after autotransplantation is shown in Figure 1A. Comparison of the groups based on the MMRM demonstrated that all oxygenated HMP groups, independent of the start time of oxygenation were superior to standard HMP without additional oxygen. Two hours of O₂ administration at the start of HMP was superior to end-ischemic O₂ supply during HMP (*P* = .04) and equivalent to the continuous oxygenation group (22-hour HMPO₂) (*P* = .36).

The daily evolution of serum creatinine after transplantation is shown in Figure 1B. No statistically significant difference was observed between the 22-hour HMPO₂ and the initial O₂ uploading group. However, both, the oxygen uploading and continuous oxygenation groups demonstrated significantly lower serum creatinine (*P* < .05), compared with the standard HMP group during the first 4 days after transplantation. This early functional benefit was not seen for the end-preservation O₂ approach.

TABLE 1 Operative data of 25 pigs in an ischemia-reperfusion autotransplant model according to the study group

Variable	2-h HMPO ₂ + 20-h HMP (<i>n</i> = 6)	20-h HMP + 2-h HMPO ₂ (<i>n</i> = 5)	22-h HMP (<i>n</i> = 6)	22-h HMPO ₂ (<i>n</i> = 8)	<i>P</i> value
Weight pig, kg	44.28 ± 2.57	43.60 ± 1.96	39.23 ± 2.00	43.83 ± 7.30	.1906
Left nephrectomy procedure time, h:m	3 h 35 ± 0 h 21	3 h 35 ± 0 h 16	3 h 16 ± 0 h 31	3 h 31 ± 0 h 26	.5214
Weight left kidney, mg					
Before preservation	108 ± 8	113 ± 11	100 ± 13	109 ± 18	.4606
After preservation	149 ± 11	162 ± 27	151 ± 22	149 ± 30	
Perfusate pO ₂ , mm Hg					
(a) Inline measured at 4°C					
At start HMP	495 ± 111	148 ± 3	106 ± 3	NA	.0002
At end HMP	134 ± 3	495 ± 65	8 ± 1	NA	<.0001
(b) Perfusate samples analyzed and recalculated at 37°C					
At start HMP	617 ± 118	208 ± 21	165 ± 9	697 ± 84	<.0001
At end HMP	219 ± 13	789 ± 14	68 ± 3	717 ± 56	<.0001
Right nephrectomy and autotransplant procedure time, h:m	3 h 30 ± 0 h 18	3 h 00 ± 0 h 04	3 h 52 ± 0 h 54	2 h 54 ± 0 h 45	.0135
Preservation time, h:m	22 h 14 ± 0 h 17	22 h 04 ± 0 h 20	22 h 40 ± 0 h 36	20 h 51 ± 0 h 57	.1777
Anastomotic time, min	24 ± 2	24 ± 2	32 ± 4	26 ± 3	.0017

Note: Data are given as the mean ± SD.

Abbreviations: HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion.

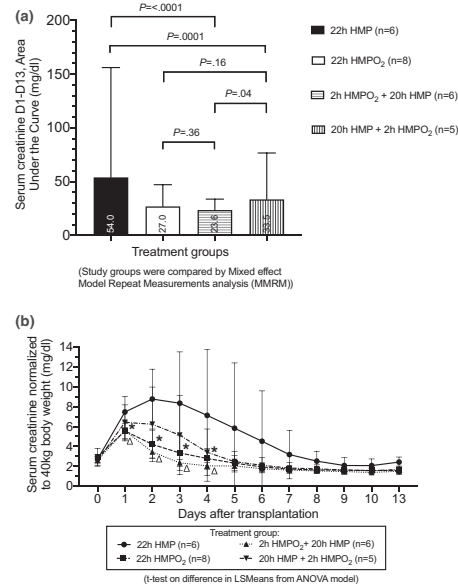


FIGURE 1 A, Area under the curve analysis of serum creatinine from day 1 until 13 after transplantation according to the study group demonstrating that oxygenated HMP, independent of the start time of oxygenation is superior to standard HMP. B, Evolution of daily postoperative serum creatinine in all study groups demonstrating superiority of the 22-h HMPO₂ and 2-h HMPO₂ + 20-h HMP group compared with standard HMP during the first 4 d after transplantation. Significance of $P < .05$ between 22-h HMP and 22-h HMPO₂ group is indicated with *. Significance of $P < .05$ between 22-h HMP and 2-h HMPO₂ + 20-h HMP group is indicated with Δ . HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion

Tubular reabsorption of sodium was transiently disturbed in all study groups after transplantation but demonstrated a faster decrease to baseline values especially during the first three postoperative days in all oxygenated machine perfusion strategies compared with standard HMP (Figure 2A). The AUC of the fractional excretion of sodium demonstrated no significant differences between the study groups (Figure 2B). Similar findings were observed for the urinary protein creatinine ratio (Figure 2C,D).

3.3 | Evolution of postoperative serum AST and LDH

In the study groups, the peak of serum AST was observed at day 1 after kidney transplantation and dropped immediately afterwards showing no differences between the study groups. The peak of serum LDH in the study groups was also observed at day 1 after

transplantation and dropped immediately afterwards into a normal range 6 days after transplantation, except for the initial O₂ uploading group where serum LDH was significantly higher during follow-up compared with all other study groups (Figure S1).

3.4 | Impact of perfusate pO₂ levels on renal flow and resistance during machine perfusion

Continuous pO₂ measurements during HMP are shown in Figure 3. Active oxygenation independent of the start time during HMP results in a significantly higher renal flow during machine perfusion compared with standard HMP without additional oxygenation (Figure 4A,B). Accordingly, the inverse findings were observed for renal resistance (Figure 4C,D).

3.5 | Perfusate glucose, lactate dehydrogenase, and aspartate transaminase

No differences in perfusate glucose and LDH were detected at the end of machine perfusion between the study groups. Perfusate AST at the end of HMP was significantly higher in the 2-hour HMPO₂ + 20-hour HMP group compared with the 22-hour HMP ($P = .003$) and 22-hour HMPO₂ group ($P = .01$) (Figure S2).

3.6 | Quantification of metabolite concentrations in perfusate

¹H-NMR analysis was used to determine the concentrations of metabolites in the circulating perfusate at the perfusion start, at 2 hours, and at the end in both experimental groups and only at the end of machine perfusion in both historical control groups (Tables S1-S3). All oxygenation strategies resulted in significantly lower concentrations of circulating metabolites lactate and succinate at the end of machine perfusion, compared with nonoxygenated HMP. These effects were most pronounced for the continuous and initial oxygenation groups with lower concentrations of lactate ($P = .01$) and succinate ($P = .03$) compared to oxygenation at the end of perfusion (Figure 5A,B).

FMN release during machine perfusion detected by fluorescence was significantly lower during the first 2 hours of machine perfusion in the initial O₂ uploading HMP group compared with the standard HMP group and end preservation-oxygenated HMP group (Figure 6).

3.7 | Oxidative stress and metabolic evaluation on preservation and reperfusion biopsies

Western blot analysis was used to determine protein expression of HO-1, SOD1, Nrf2, and HIF-1 α in both experimental groups

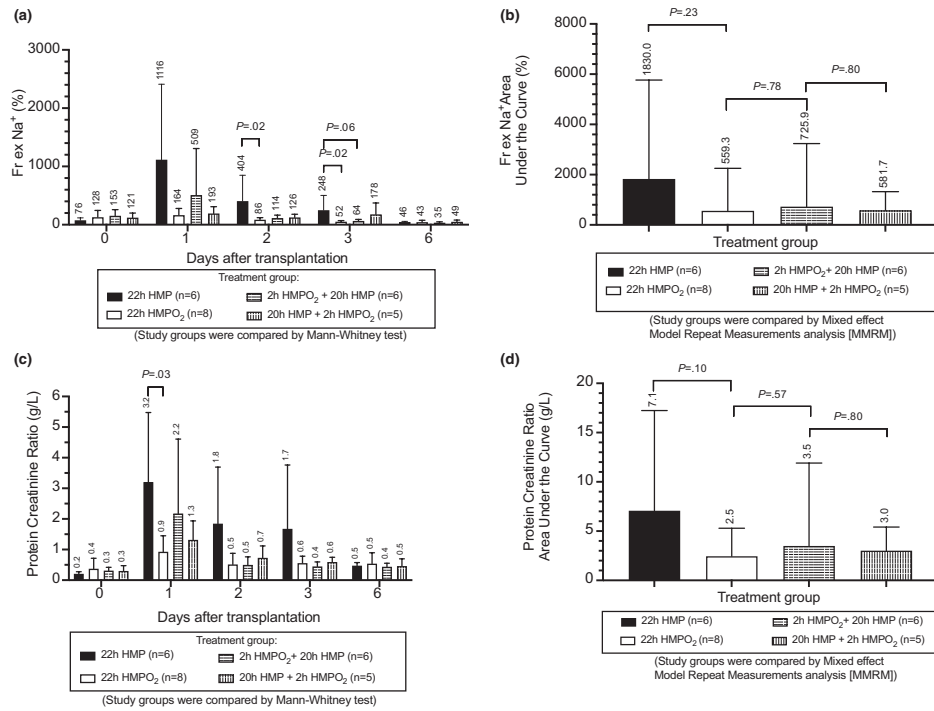


FIGURE 2 The daily evolution and the area under the curve analysis of the urinary fractional excretion of sodium and protein creatinine ratio according to the study groups (A, B, C, and D, respectively). Fr ex Na⁺, fractional excretion of natrium; HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion

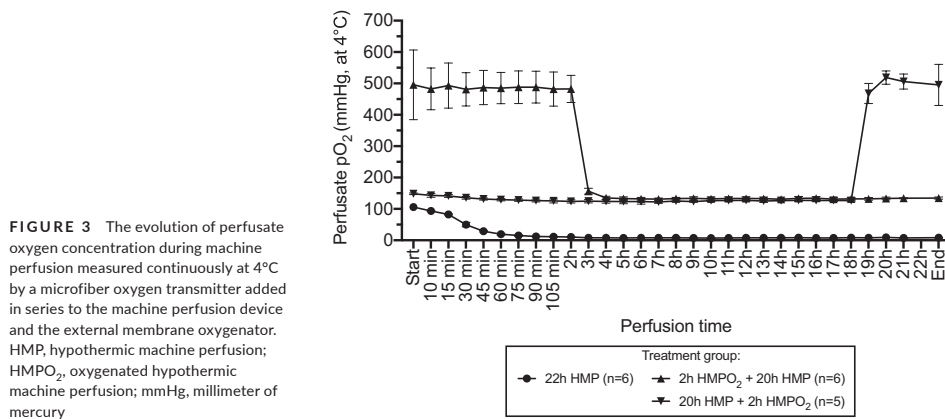


FIGURE 3 The evolution of perfusate oxygen concentration during machine perfusion measured continuously at 4°C by a microfiber oxygen transmitter added in series to the machine perfusion device and the external membrane oxygenator. HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion; mmHg, millimeter of mercury

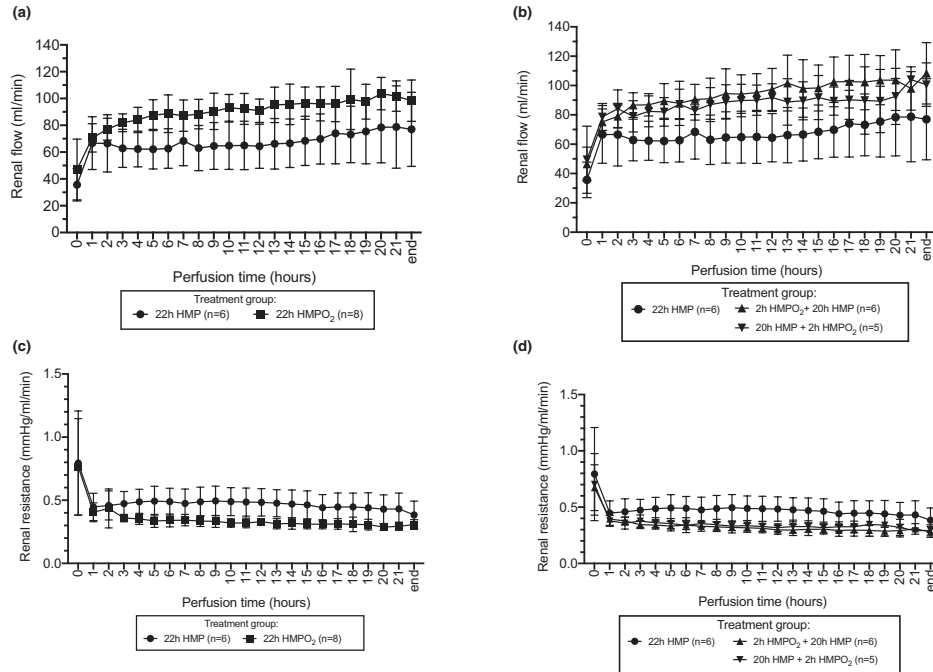


FIGURE 4 Influence of oxygen supply on ex vivo renal flow and renal resistance during continuous oxygenated HMP (A and C, respectively) and initial or end-ischemic O₂ uploading during HMP (B and D, respectively) compared with standard HMP. HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion compared with standard HMP

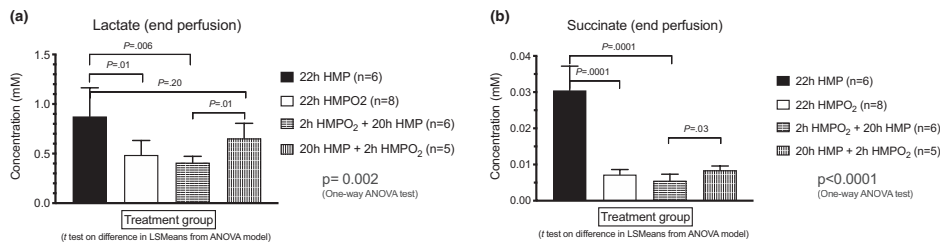


FIGURE 5 Concentrations of lactate and succinate in the circulating perfusion fluid measured by proton nuclear magnetic resonance, demonstrating the influence of oxygen independent of start time in all oxygenated machine perfusion groups compared with standard HMP. HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion compared with standard HMP

compared with the standard HMP group (Figure 7). We did not observe any difference in SOD1 and HO-1 expression comparing the study groups at the end of cold preservation and after 5 minutes of reperfusion. The SOD1 expression in the O₂ uploading group was significantly higher after 45 minutes of reperfusion compared with both other study groups. The Nrf2 expression was significantly

lower after preservation in the O₂ uploading HMP group compared with the standard HMP group ($P = .001$). The Nrf2 expression after 5 minutes of reperfusion in situ after implantation was significantly lower in the initial O₂ uploading and the end-ischemic O₂ uploading group compared with the standard HMP group ($P = .004$ and $.002$, respectively). No difference of HIF-1 α expression was

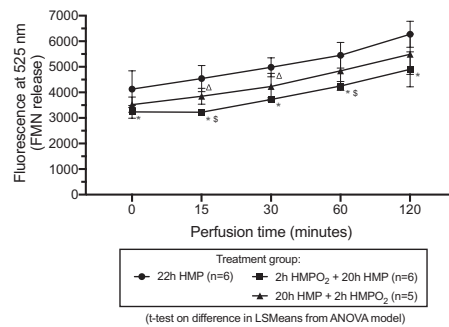


FIGURE 6 Flavin mononucleotide detection in the perfusate by fluorescence at 525 nm demonstrating significantly lower values during the first 2 h of machine perfusion inversely correlated with the perfusate oxygen concentration according to the study group. FMN, flavin mononucleotide; HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion. Significance of $P < .05$ between 22-h HMP and 2-h HMPO₂ + 20-h HMP group is indicated with *. Significance of $P < .05$ between 22-h HMP and 20-h HMP + 2-h HMPO₂ group is indicated with Δ . Significance of $P < .05$ between the 2-h HMPO₂ + 20-h HMP and 20-h HMP + 2-h HMPO₂ is indicated with δ

observed between study groups at the end of preservation and after reperfusion.

No differences in lactate level were observed in biopsies obtained during preservation and after reperfusion comparing both experimental groups and the standard HMP group (Figure 8A) by LC-ESI-MS analysis. Succinate and glutamate levels measured at the end of the cold preservation were significantly lower in initial and end preservation-oxygenated groups compared with the standard 22-hour HMP group with no additional oxygen ($P = .04$ and $P = .04$ and $P = .02$ and $P = .02$, respectively for succinate and glutamate) (Figure 8B,C). The ATP levels, detected at the end of cold perfusion were significantly higher in kidneys receiving the oxygen initially compared with the standard 22-hour HMP ($P = .02$). No differences were observed between the standard HMP and the 20-hour HMP + 2-hour HMPO₂ group ($P = .18$) (Figure 8D). Brief initial or end-preservation O₂ uploading during HMP resulted in an 8- and 2-fold ATP level increase at the end of cold preservation compared with standard HMP, respectively. No significant differences were observed between both study groups and the standard HMP group regarding ADP, AMP, and NADH levels at the end of cold perfusion (Figure 8E-H). At 5 and 45 minutes after in situ reperfusion, NAD⁺ was significantly higher in kidneys treated with oxygen initially when compared to standard 22-hour HMP group ($P = .001$ and $P < .0001$, respectively) (Figure 8H).

3.8 | Histology: light microscopy

Kidney biopsies were assessed at the beginning and the end of cold perfusion and also 13 days after implantation according to the Banff

criteria¹⁵ and the acute tubular injury score of SA Hosgood.¹⁶ No differences were observed between all study groups (data not shown) at each biopsy time point.

4 | DISCUSSION

This ischemia-reperfusion porcine kidney autotransplant model is the first animal study to evaluate the impact of the start time of active oxygenation during continuous HMP on early graft recovery. The aim was to determine the optimal conditions for hypothermic kidney preservation to provide first an effective oxygenation and secondly an easy approach to not complicate logistics during organ donation and transport. Two-hour perfusate oxygenation, right at the start of HMP led to significantly improved early renal function when compared with 22-hour standard HMP without additional oxygen supply. Interestingly, perfusate oxygenation at the start of HMP achieved comparable outcomes as seen with continuous oxygenation during HMP. The established metabolic profile obtained in perfusate (ie, lactate, succinate, and FMN) and kidney tissues (ie, succinate, glutamate, ATP, Nrf2, and HIF-1 α) suggests that a brief O₂ uploading at the start of HMP could be an easy and more beneficial preservation strategy to protect mitochondria and improve early graft function as compared with standard HMP without additional oxygenation and O₂ supply only at the end of the HMP period.

There is considerable evidence that mitochondrial succinate accumulation originating from the citric acid cycle (CAC) during ischemia is one main contributor for superoxide generation by reverse electron transport (RET) during subsequent in vivo reperfusion under normothermic conditions.¹⁷⁻¹⁹ Dutkowski et al²⁰ described this mechanism assessed during different human liver perfusion strategies. In addition to the functional benefits of cold oxygenation, our study provides corroborative metabolic evidence in both perfusate (ie, lactate, succinate, and FMN) and tissue analysis (ie, succinate, glutamate, and ATP levels) that similar pathological processes occur during kidney perfusion that can be improved via oxygenation. The curious observation that massive changes in end preservation ATP levels between the different HMP groups did not lead to reciprocal changes in AMP levels might warrant further investigation.

The effects of oxygen during different cold perfusion strategies on mitochondria are illustrated in Figure 9. The main source of in vivo cellular ATP is mitochondrial oxidative phosphorylation, which is dependent on oxygen as the final electron acceptor (Figure 9A).^{19,21-25} Complex I composed of 45 subunits contains one molecule of tightly, but non-covalently bound FMN and eight different iron-sulfur clusters.²⁴ Flavin serves as the primary electron acceptor from NADH, and a series of iron-sulfur clusters provide a pathway for the forward electron transfer to the bulk ubiquinone (Q).²⁴ During hypoxia, forward electron transport (FET) is interrupted with a number of pathological sequelae due to RET (accumulation of NADH, succinate and purine metabolites, FMN release [FMNH₂], and ATP/ADP decrease) (Figure 9B).^{19,20,24-26} Cold machine preservation solutions were originally developed

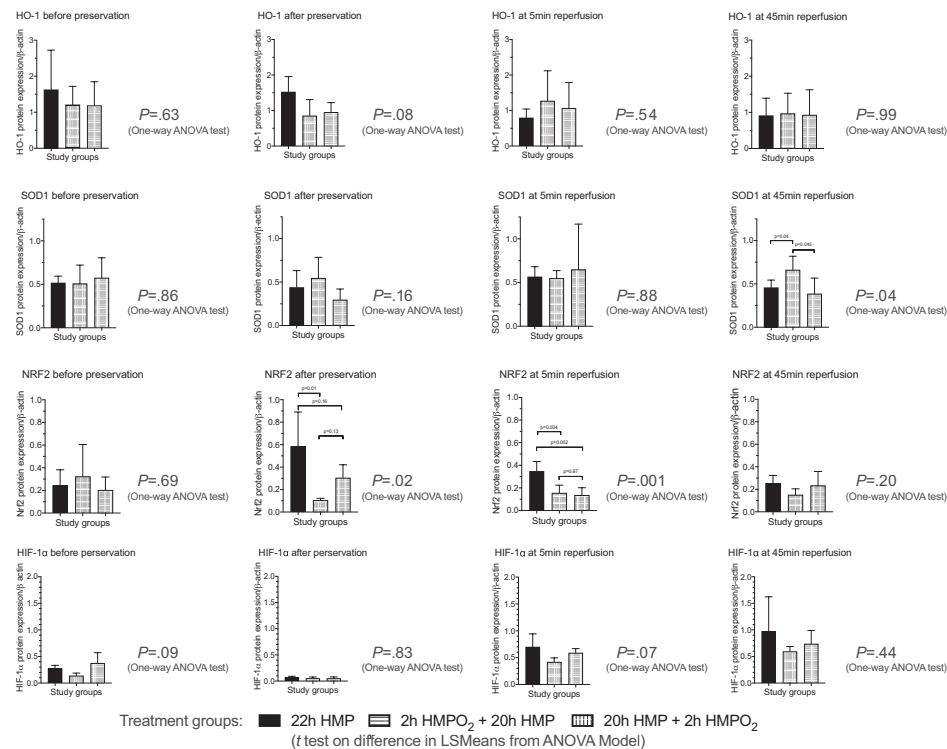


FIGURE 7 Oxidative stress-related protein expression analysis. Oxygenation during HMP reduces Nrf2 expression at the end of preservation and during early reperfusion compared to standard HMP. HIF-1α, hypoxia-inducible factor 1α; HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion; HO-1, heme oxygenase 1; Nrf2, nuclear factor erythroid 2-related factor 2; SOD1, superoxide dismutase 1

for nonoxygenated HMP and do not contain an oxygen carrier. Therefore, tissue oxygenation is provided by diffusion. In this study we observed that the diffusion of oxygen into perfusion fluid early during the HMP promotes physiological mitochondrial processes with evidence of FET activity, as seen by lower NADH and succinate levels and subsequent less FMN release, and higher ATP resynthesis, compared with standard HMP or end-preservation O₂ supply during HMP (MODE 3 of mitochondrial operation according to Murphy) (Figure 9C).^{20,25} During early in vivo reperfusion, FET generates ATP in the presence of oxygen. However, rapid consumption of accumulated succinate generates excessive ubiquinol (QH₂), which impairs further FET. Such high succinate levels in combination with an acidotic milieu during ischemia result in RET at complex I with subsequent ROS production (Figure 9D).^{19,27} Recent literature suggests that it is not the accumulated ischemic succinate but more likely the reduced

or semireduced flavin located at the nucleotide-binding site that appears as the direct source of ROS production at complex I.²⁴ Reduced flavin (FMNH₂) can nonenzymatically react with molecular oxygen and generate ROS and oxidized flavin, which may rebind at complex I and recover the enzymatic activity during later reperfusion.²⁴ FMN determination during HMP by simple fluorescence is far easier and faster than any currently available succinate measurement and could therefore potentially be used as real-time surrogate marker to assess the metabolic status and predict IRI in solid organs. Indeed, Muller et al²⁸ described the use of FMN to predict liver function already during hypothermic oxygenated liver perfusion before implantation. We do realize that the perfusate FMN measurement by fluorescence detects only released FMN dislocated from complex I and does not detect the FMN that is still bounded in complex I. Because the proportion of released FMN originated from ischemic cell lyses is probably related to the

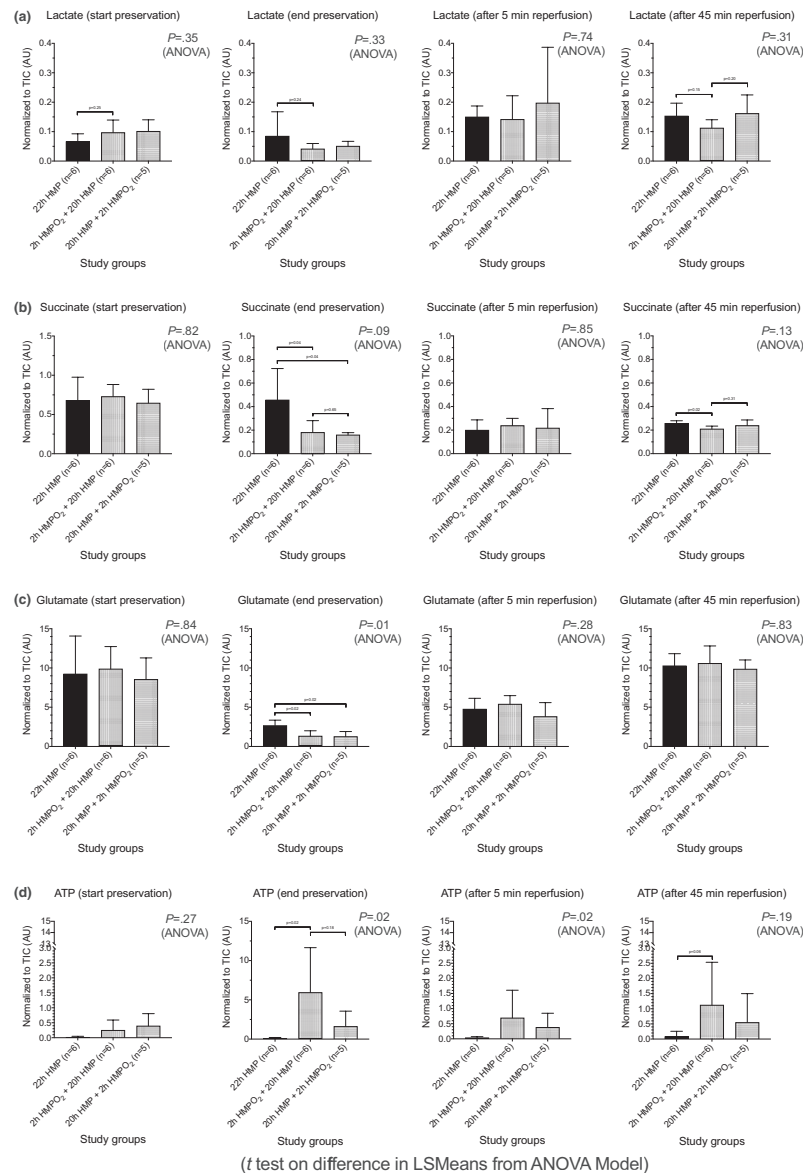
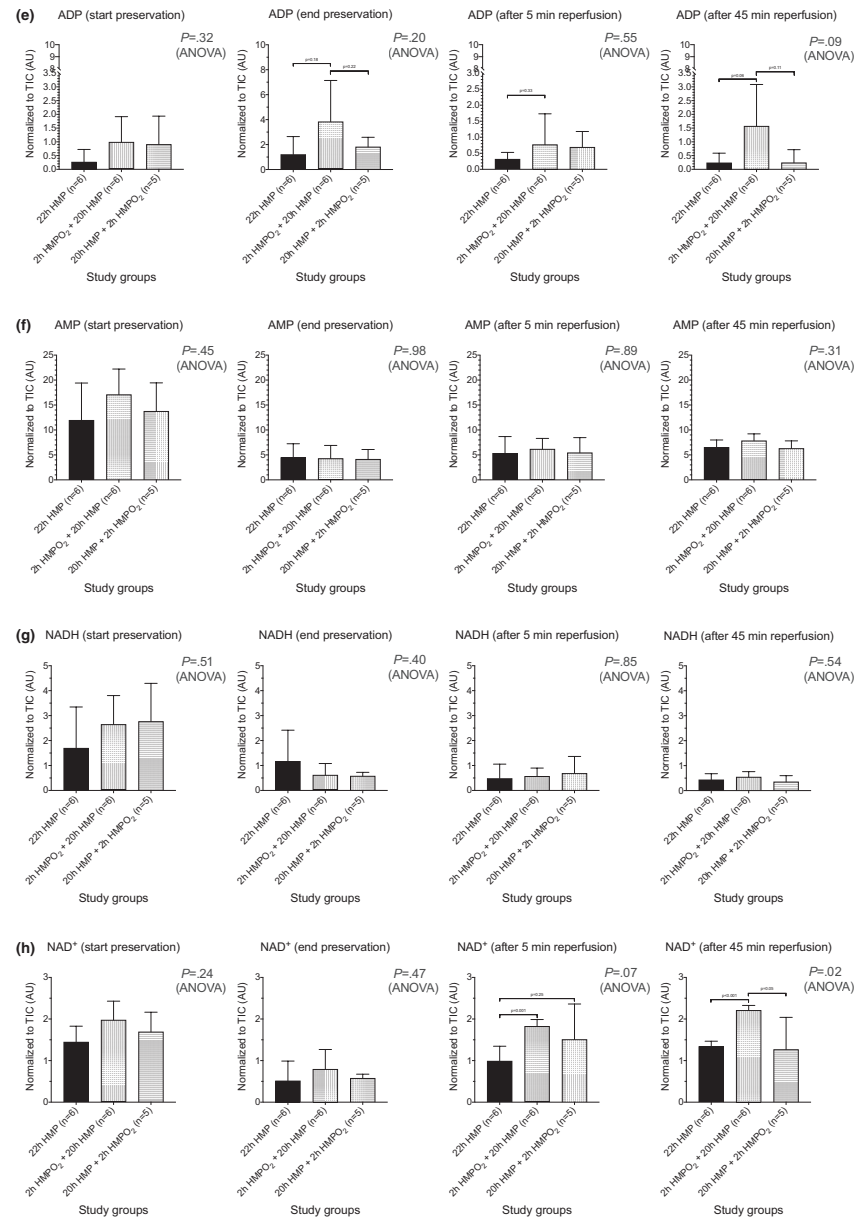


FIGURE 8 Metabolic profile analysis on preservation and reperfusion biopsies by liquid chromatography electrospray ionization mass spectrometry demonstrates that active oxygenation during HMP better supports aerobic metabolism, in particular the central major metabolites succinate and glutamate and the end product ATP. ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion



(*t* test on difference in LSMeans from ANOVA Model)

FIGURE 8 Continued

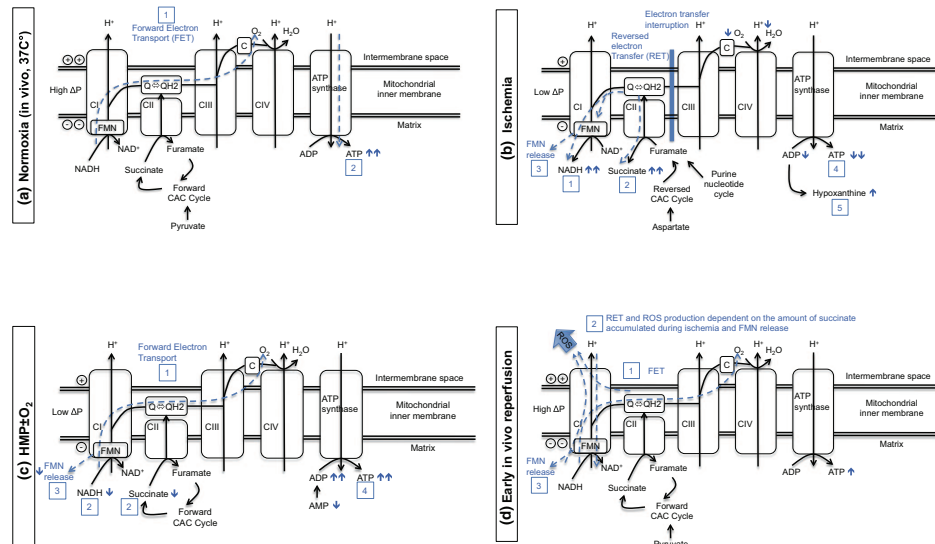


FIGURE 9 The effect of different kidney oxygenated HMP strategies. A, In vivo, during physiologic oxygenated conditions at 37°C, forward electron transport (FET) (dotted line) (1) drives the proton pumps (CI, CIII, CIV) against a charge gradient at the oxidative phosphorylation chain and proton back flow drives ATP synthase to generate ATP (dotted line) (2).^{19,25} B, During ischemia, major metabolic alterations occur at the mitochondrial level in the kidney graft.^{18,19} In the absence of oxygen, electron transfer is interrupted and first NADH (1) and second citric acid metabolite succinate (2) increase because both act now as electron carrier to allow CI continuously pump protons during ischemia. Also FMN release (3) at CI results from over-reduction of flavin via reversed electron transfer (RET).^{24,26} Consequently, ATP and ADP decrease (4) and purine metabolites increase (5) (MODE 1 of mitochondrial operation according to Murphy).^{19,25} C, During HMP conditions, mitochondrial respiratory function is significantly slowed down preventing the accumulation of electron overload at CI and CII. The degree of FET during HMP depends on the perfusate oxygen concentration (1), in particular during the first 2 h of HMP and results in a decrease of succinate and NADH (2) and less FMN release (3) and an increase of ADP/ATP regeneration (4) (MODE 3 of mitochondrial operation according to Murphy).^{20,25} D, During early, in vivo reperfusion, FET (1) generates ATP. However, in case of too much accumulated mitochondrial succinate, rapid consumption of accumulated succinate generates too much ubiquinol (QH₂), which impairs further FET. In combination with acidic pH from ischemia this results in a RET at CI (2).^{19,27} Reduced or semireduced FMN located at the nucleotide-binding site is the direct source of ROS in CI during RET but reduced FMN can also nonenzymatically react with molecular oxygen producing ROS.²⁴ FMN, flavin mononucleotide; CI-IV, mitochondrial complex I-4 [Color figure can be viewed at wileyonlinelibrary.com]

duration of machine perfusion, we focused on FMN measurement during the first 2 hours of HMP, which correlated well with initial graft function in our study.

Nrf2 is a transcription factor that coordinates the basal and stress-inducible activation of antioxidants as well as cytoprotective genes and therefore acts as a protective transcription factor in IRI.^{29,30} In our study, brief initial oxygenation resulted in the lowest Nrf2 expression at the end of HMP and following early in situ reperfusion, suggesting a reduced oxygen stress with O₂ uploading. Hypoxia-inducible factor is a heterodimeric protein composed of an oxygen-regulated alpha (α) and a constitutively expressed beta (β) subunit.³¹⁻³⁵ HIF-1α accumulation during renal ischemia has been widely reported.³⁶ In contrast, we observed a drop in HIF-1α expression in all conditions measured right after preservation attest of the high sensitivity to experimental procedures

and precludes from any interpretation on subsequent variations. We found that HIF-1α levels were lowest in the initial oxygenation group following reperfusion. This might be explained by the difference in pO₂ during the first 2 hours of HMP comparing both experimental groups.

We recognize that there are a number of limitations inherent to this porcine autotransplant.⁶ The use of historical control groups might have introduced a relevant bias due to slightly shorter anastomosis time in the experimental study groups compared with one of the historical groups but was recommended by the ethics committee to reduce animal numbers and suffering. However, because total preservation time was comparable between all study groups we do not expect to have an important influence on the outcome measures. Tubular injury measured as fractional excretion of urinary sodium and urinary protein-to-creatinine ratio is commonly used in

porcine animal models to evaluate the impact of IR^{1,6,37}. Despite efforts to ensure homogeneity in this animal model, several outcome measures (eg, urinary sodium and ATP) demonstrated significant variations and thus small differences between study groups may not have been identified.

An interesting observation was that even without oxygen supply to the external oxygenator a greater perfusate pO₂ was observed compared with standard HMP (Figure 3A). This mechanism is presumably due to passive exposure to room air through fibers of the membrane oxygenator. As a consequence the perfusate pO₂ in both initial and end oxygenation groups remained static during 20 hours without active oxygenation.

Practically, both brief initial and end-preservation oxygenation might be easy to implement in clinical practice. Kidneys originated from donors after circulatory death with more ATP debt at the start of preservation, compared with kidneys originated from brain death donors, probably benefit more from brief initial O₂ uploading at the donor hospital. This eliminates the need for an external oxygen source during transport. Concerning end-preservation oxygenation during HMP, it is unknown if the beneficial effect of oxygen maintains using shorter preservation periods as in most clinical programs compared with the model used in this study.

In conclusion, brief initial O₂ uploading during standard HMP might be an easy and more effective preservation strategy to maintain the aerobic metabolism and protect mitochondria with subsequently improved early renal function, particularly, when compared with standard HMP or end-ischemic O₂ supply. Although initial oxygen supply was not superior compared to continuous oxygenation, such short cold perfusion with oxygen could be of interest because it eliminated the need for an oxygen source for HMP during kidney transport.

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DISCLOSURE

The authors of this manuscript have conflict of interests to disclose as described by the *American Journal of Transplantation*. The LifePort Kidney Transporter, the Perfusion Circuits, and the oxygenators were supplied by Organ Recovery Systems (Diegem, Belgium).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Summary Paper 3

Aim of the study:

The aim of the study was to evaluate the optimal start time of oxygenation during continuous hypothermic machine perfusion (HMP) in a porcine ischemia-reperfusion DCD autotransplant model.

Main conclusions:

- The brief initial and continuous oxygenated HMP groups were associated with superior graft recovery compared to either standard HMP without active oxygenation or kidneys oxygenated only at the end of HMP.
- This correlated with significant metabolic difference in perfusion fluid (eg. lactate, succinate, flavin mononucleotide (FMN)) and tissues (eg. succinate, adenosine triphosphate, hypoxia inducible factor 1 alpha, nuclear factor erythroid 2-related factor 2) suggesting superior mitochondrial preservation with brief initial or continuous oxygenation during HMP compared with either standard HMP without active oxygenation or kidneys oxygenated only at the end of HMP.
- Flavin mononucleotide (FMN) has the potential to be a real time marker during HMP to predict kidney ischemia-reperfusion injury and delayed graft function.
- Brief initial oxygen uploading during HMP at the procurement site might be an easy and effective preservation strategy to maintain aerobic metabolism, protect mitochondria, and achieve an improved early renal graft function compared with standard HMP without active oxygenation or oxygen supply shortly at the end of HMP preservation.

Personal contributions
Results: Paper 4

Paper 4: Brief bubble and intermittent surface oxygenation is a simple and effective alternative for membrane oxygenation during hypothermic machine perfusion in kidneys.

Personal contributions
Results: Paper 4



OPEN

Brief Bubble and Intermittent Surface Oxygenation Is a Simple and Effective Alternative for Membrane Oxygenation During Hypothermic Machine Perfusion in Kidneys

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Background. The aim of this feasibility study was to determine an alternative oxygenation technique (easy, cheap, and compatible with air transport) for membrane oxygenation during hypothermic machine perfusion (HMP) to improve early graft function in a porcine ischemia-reperfusion autotransplant model. **Methods.** The left kidney of a ± 40 -kg pig was exposed to 30 minutes of warm ischemia before 22 hours of preservation and autotransplantation. In the experimental group, oxygenation of the perfusate during HMP was obtained by direct bubble and 30-minute surface oxygenation at start and 1-hour end ischemic ($n=4$) and outcome measures compared with historical HMP without active oxygenation ($n=6$), 22-hour continuous oxygenated HMP (HMPO₂) ($n=8$), and 2-hour HMPO₂+20-hour HMP ($n=6$) using membrane oxygenation in both historical oxygenated control groups. **Results.** Brief bubble and 30-minute surface oxygenation of the perfusate effectively maintained supraphysiological Po₂ levels during the first 2 hours of HMP with improved flow dynamics. Although the metabolic profile of the perfusate (ie, flavin mononucleotide) and tissue (ie, glutamate, ATP) after brief O₂ uploading at the start of HMP seemed to be slightly better with the use of a membrane oxygenator compared with bubble and interrupted surface oxygenation, both techniques yielded similar, superior early graft function when compared with HMP without active oxygenation. **Conclusions.** The data presented in this feasibility study support the conclusion that brief bubble and intermittent surface oxygenation could be an alternative oxygenation technique during HMP to achieve an improved kidney graft function compared with HMP without active oxygenation and similar functional outcome when compared with membrane HMPO₂.

(*Transplantation Direct* 2020;6: e571; doi: 10.1097/TXD.0000000000001016. Published online 11 June, 2020.)

The protective effect of supplemental oxygenation (continuous or end ischemic) during hypothermic machine perfusion (HMP) has been demonstrated in preclinical studies.¹⁻⁶ In addition, aerobic mechanisms were better supported under high oxygen concentrations during kidney machine perfusion.^{1,7-9} Our group demonstrated in an ischemia-reperfusion porcine kidney autotransplant model⁶ that brief O₂

uploading by a membrane oxygenator at the start of HMP during 2 hours is an effective preservation strategy for protecting mitochondria.¹⁰ Such a preservation strategy yielded superior early graft function compared with standard, non-oxygenated HMP (non-HMPO₂) or briefly administering oxygen at the end of HMP preservation. Brief O₂ uploading resulted in similar early graft function as observed with continuous oxygenation during HMP.¹⁰ These oxygenated

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preservation strategies could help to decrease the harmful effects of ischemia-reperfusion injury, especially in an era of frequent high-risk donor organ utilization, with associated morbidity.¹¹⁻¹⁴

However, the optimal administration technique for oxygen during HMP has not yet been explored. Eliminating a membrane oxygenator as well as an oxygen source during organ transport could reduce the cost of HMPO₂ and facilitate air transport.

We therefore evaluate the use of direct bubble and intermittent surface oxygenation during continuous HMP as alternative method for brief O₂ uploading by a membrane oxygenator on early graft function in a pig kidney ischemia-reperfusion autotransplant model. We further measure renal flow, resistance, and concentration of Po₂ in the perfusate during machine perfusion and assess tubular function. Biomarkers and metabolic profiles are obtained from perfusate and renal biopsies.

MATERIALS AND METHODS

Animals

Female Belgian Landrace pigs of approximately 40 kg were used. Experiments were performed according to international guidelines, after approval by the local Ethical Committee for Animal Care. The animals were cared according to Belgian laws on experimental animal welfare.

Study Design

A standardized ischemia-reperfusion porcine kidney autotransplant model was used.⁶

Anesthetic and Surgical Protocol

Anesthetic and surgical protocols were described previously.^{6,9,10} The left kidney was removed after 30 minutes of warm ischemia induced by vascular clamping and subsequently flushed with 200 mL of Kidney Perfusion Solution-1 (Organ Recovery Systems, Diegem, Belgium). In the experimental group, oxygenation of the perfusate during HMP was obtained by direct bubble and 30-minute surface oxygenation at the start and 1-hour end ischemic (22-hour HMP + interrupted surface oxygenation) (n=4). Historical control groups were 22-hour standard HMP (n=6) with no additional oxygenation, 22-hour continuous HMPO₂, and 2-hour HMPO₂ + 20-hour HMP (n=6).¹⁰ Perfusate oxygenation was obtained by membrane oxygenation in both historical oxygenated control groups. Following preservation, the left kidney was autotransplanted into the right renal bed after right nephrectomy.⁶

The surviving animals were euthanized on days 7 and 13 after transplantation by a lethal intravenous injection of T61 (Intervet International; MSD Animal Health, Boxmeer, The Netherlands) under general anesthesia, in the experimental and the historical control groups, respectively.

Machine Perfusion

The LifePort Kidney Transporter (Organ Recovery Systems, Diegem, Belgium) was used for all machine perfusion groups. The Kidney Perfusion Solution-1 was pumped through the kidney at a pressure of 30 mmHg. The temperature of the kidney and the circulating perfusate was maintained at 2°C to 8°C.

Oxygenated Machine Perfusion

Carbogen (95% O₂/5% CO₂) at 500 mL/min was used for oxygen supplementation at 90% in the experimental group to have identical conditions when compared with the historical oxygenated groups. Two different oxygen administration techniques were compared.

Oxygenation via Membrane Oxygenator

The perfusate circuit of the LifePort Kidney Transporter was modified to include a membrane oxygenator (Dideco Kids D100 neonatal oxygenator; Sorin Group, Mirandola, Italy).^{6,9,10}

Bubble and Surface Oxygenation

Oxygen uploading of the perfusate was obtained by bubble oxygenation in the reservoir during the circuit setup (wash phase) before connecting the kidney to the device. This was realized by insufflating the carbogen directly to perfusion solution in the reservoir via the submerged perforated O₂ tubing segment (Figure S1, SDC, <http://links.lww.com/TXD/A255>). Bubble oxygenation was switched to surface oxygenation at the start of renal perfusion. Surface oxygenation was only applied during the first 30 minutes of kidney perfusion to mimic the clinical setting of the perfused kidney leaving the donor hospital. Surface oxygenation was restarted at the end of the machine perfusion during 1 hour before autotransplantation to mimic the clinical setting of the perfused kidney arriving in the transplant center.

Outcome Measures

The primary outcome measure was early graft function (measured as daily serum creatinine). The secondary outcome measures were tubular function (measured by urinary fractional excretion of sodium and protein creatinine ratio), serum markers (lactate dehydrogenase [LDH] and aspartate transaminase [AST]), renal flow and resistance during HMP, Po₂ during machine perfusion, metabolic perfusate and tissue profile, and graft histology.

Samples and Analyses

Renal Flow and Resistance Measurement During HMP and Perfusate Analyses

The machine perfusion device continuously registers renal flow and resistance. Perfusate samples were taken at start, 15 minutes, 30 minutes, 60 minutes, 120 minutes, and at the end of machine perfusion. Perfusate Po₂ was continuously measured during perfusion at 4°C by a microfiber oxygen transmitter (Oxy-4 micro; Precision Sensing GmbH, Regensburg, Germany). In the historical 22-hour HMPO₂, perfusate samples and Po₂ measurement were only performed at start and end perfusion. Glucose, AST, and LDH analyses were performed by a dry chemistry analyzer Fuji Dri-Chem NX500 (Fujifilm Corporation, Tokyo, Japan). Perfusate samples were analyzed by fluorescence at 525 nm to detect the amount of flavin mononucleotide (FMN) release.

Blood and Urine Analysis

Venous blood samples and urine samples were collected daily during follow-up for biochemical analysis (eg, creatinine, urea, sodium, glucose, AST, LDH, and proteinuria) determined with methods of the clinical routine.

Tissue Sampling and Analysis

Kidney biopsies were taken at 5 time points. Wedge biopsies were taken before preservation, after 22 hours of preservation, and before euthanasia. Needle biopsies were taken after 5 and 45 minutes reperfusion. Biopsies were stored both fresh at -80°C pending metabolic analysis and also fixed in acidified formal alcohol, dehydrated, and embedded in paraffin wax for histology.

Metabolites (lactate, succinate, glutamate, AMP, ADP, ATP and oxidized and reduced nicotinamide adenine dinucleotide [oxidized nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide, respectively]) were extracted from frozen kidney biopsies by methanol-chloroform extraction and analyzed by liquid chromatography coupled to electrospray ionization mass spectrometry

using a method based on Coulier et al.¹⁵ Metabolite quantification was performed as described by Veiga-da-Cunha et al.¹⁶

Sections of 4 μm were cut and stained with hematoxylin and eosin for evaluation using light microscopy. Sections were scored blindly assessing changes according to the histopathologic consensus criteria for preimplantation kidney biopsies¹⁷ and an acute tubular injury score described by Hosgood et al.¹⁸ to assess changes in 4 morphological categories: tubular dilatation, tubular debris, vacuolation, and infiltration.

Statistical Methods

The study follows a parallel design with 4 active groups. No imputation was done so all analyses were based on the observed cases only.

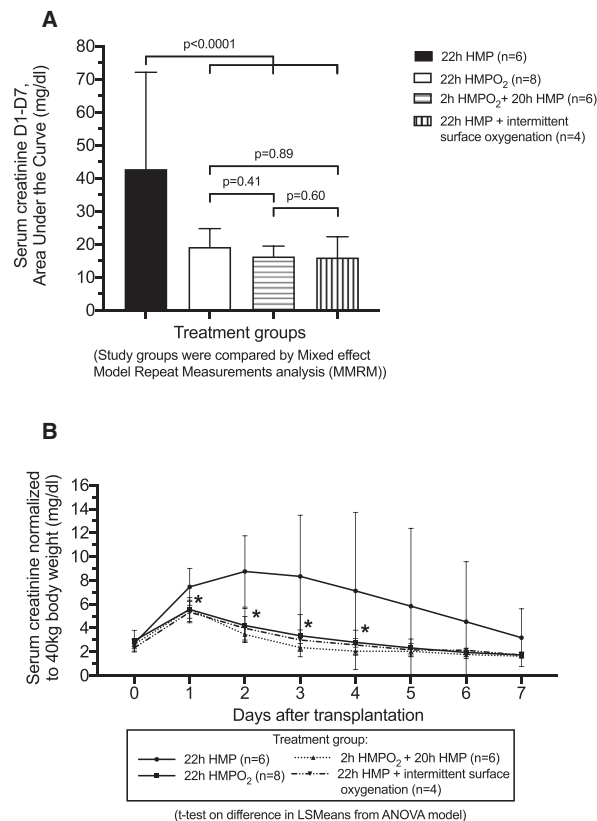


FIGURE 1. Evolution of serum creatinine. Area under the curve analysis of serum creatinine from days 1 until 7 after transplantation according to the study group demonstrating that all oxygenated HMP groups, independent of oxygen administration technique and duration of O_2 supply during machine perfusion, are superior to standard, nonoxygenated HMP (A). Evolution of daily serum creatinine demonstrating superiority of all oxygenated HMP groups especially during the first 4 days after transplantation compared with the standard, nonoxygenated HMP groups (B). *Significance of $P < 0.05$ between the 22-h HMPO₂, the 2-h HMPO₂+20-h HMP, and the 22-h HMP+intermittent surface oxygenation group compared with standard 22-h HMP. HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion.

The primary outcome measure of early graft function was assessed through analysis of serum creatinine over time using generalized linear model techniques. Serum creatinine values were normalized to 40 kg animal body weight before use in the analysis to correct for differences in animal and organ size. Serum creatinine was analyzed using mixed model for repeated measurements (MMRM) with factors treatment, time point, and interaction term to test for overall difference in graft function over time between groups. Individual comparison of groups was conducted using contrast analyses within the main MMRM model testing the null hypothesis that the least squares mean difference between 2 groups was zero (no mean difference). For the contrast analysis, *P* values (based on *t* test of least squares means) were reported along with a mean (SD) graph where appropriate. All secondary end point variables were similarly modeled to the primary end point. In case of variables without repeated measurement (eg, area under the curve [AUC]), the MMRM model was reduced to an ANOVA model with factor treatment. As this was a preclinical exploratory trial, no formal sample size calculations were performed, and no correction for multiplicity was foreseen. All analyses were performed using SAS 9.4 (SAS Institute, Cary, NC) and Prism 8.2.0 (GraphPad Software, San Diego, CA).

RESULTS

Operative Data, Major Complications, and Animal Survival

Twenty-eight autotransplantations were performed, and 4 were excluded from the analysis due to early death or graft-interfering complications (sudden death at day 2 [*n*=1], hydronephrosis of the right kidney diagnosed during the autotransplant procedure because of mechanical obstruction caused by the balloon of the bladder catheter [*n*=1], respiratory arrest 30 min after extubation on day 0 [*n*=1], arterial thrombosis at day 3 necessitating euthanasia [*n*=1]). Operative data of the 24 autotransplantations are presented in Table S1, SDC, <http://links.lww.com/TXD/A255>. The use of historical control groups resulted in a slightly longer anastomosis time in 2 of these 3 groups compared with the experimental study group.

Graft Function

The AUC of the serum creatinine within 7 days after autotransplantation is shown in Figure 1A. All oxygenated study groups, independent of the O₂ administration technique and duration of O₂ supply during machine perfusion, were superior to HMP without active oxygenation. No differences were observed between all oxygenated study groups.

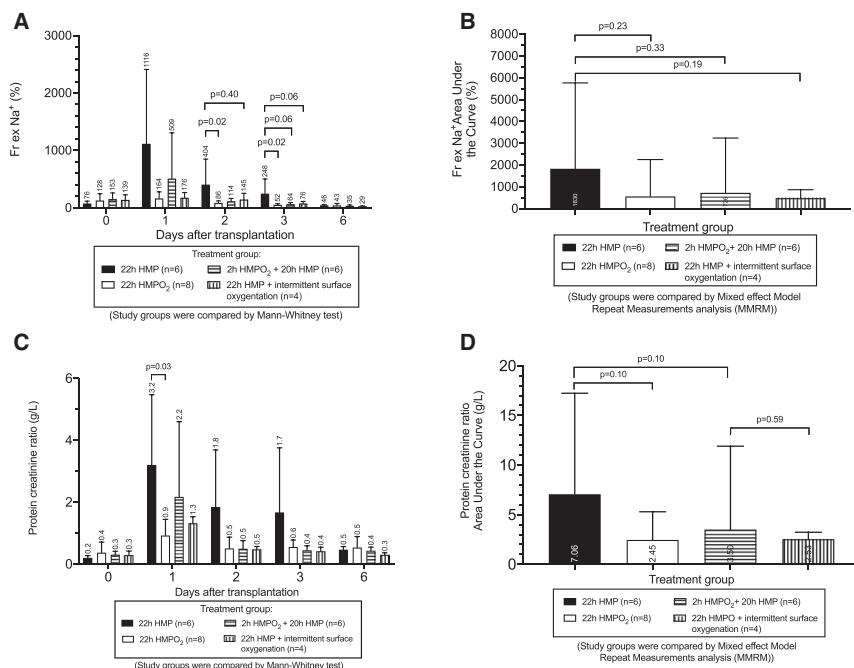


FIGURE 2. Evolution of tubular function. The daily evolution and the area under the curve analysis of the urinary fractional excretion of sodium and protein creatinine ratio according to the study groups (A and B and C and D, respectively). Fr ex Na⁺, fractional excretion of sodium; HMP, hypothermic machine perfusion; HMP+O₂, oxygenated hypothermic machine perfusion.

All oxygenated study groups demonstrated significantly lower serum creatinine ($P < 0.05$) compared with the standard, non-HMPO₂ group during the first 4 days after transplantation (Figure 1B). This early functional benefit was comparable in all oxygenated study groups.

Tubular reabsorption of sodium was transiently disturbed in all study groups early after transplantation but demonstrated a faster decrease to baseline values, especially during the first 3 postoperative days in all oxygenated machine groups compared with the standard non-HMPO₂ (Figure 2A). The AUC of the fractional excretion of sodium demonstrated no significant differences between the study groups (Figure 2B). Similar findings were observed for the urinary protein creatinine ratio (Figure 2C and D).

Evolution of Postoperative Serum AST and LDH

In all study groups, the peak of serum AST was observed at day 1 and dropped immediately afterward showing no differences between the study groups (Figure 3A). The peak of serum LDH in the study groups was observed at day 1 and dropped afterward to a normal range 6 days after transplantation, except for the 22-hour experimental group where serum LDH peaked again at day 6 to drop down the day after (Figure 3B). No significant differences in serum AST and LDH were observed between the study groups.

Impact of Oxygen Delivery Technique on Perfusate Po₂, Renal Flow, and Resistance During Machine Perfusion

During the perfusion circuit setup, perfusate oxygen concentrations of >400 and 500 mmHg at 4°C were obtained by bubble oxygenation after 20 and 30 minutes, respectively

(Figure 4A). Continuous Po₂ measurements during HMP are shown in Figure 4B. Thirty minutes of surface at the start was able to maintain supraphysiological Po₂ levels during the first 2 hours of HMP. The effect of 1-hour surface oxygenation of the perfusate at the end of HMP demonstrated a slight increase of perfusate Po₂. In contrast, prolonged oxygenation for 2 hours at the start via a membrane oxygenator kept perfusate Po₂ levels more stable when compared with shorter surface oxygenation.

Active oxygenation independent of the administration technique results in a significantly higher renal flow during machine perfusion compared with standard HMP, without additional oxygenation (Figure S2A, SDC, <http://links.lww.com/TXD/A255>). Accordingly, the inverse findings were observed for renal resistance (Figure S2B, SDC, <http://links.lww.com/TXD/A255>).

Perfusate Glucose, Lactate Dehydrogenase, and AST

No differences in perfusate glucose, LDH, and AST were detected at the end of machine perfusion between the study groups ($P = 0.16$; $P = 0.95$, $P = 0.15$, respectively) (Figure S3, SDC, <http://links.lww.com/TXD/A255>).

Perfusate FMN

FMN release during machine perfusion was significantly lower during the first 2 hours of machine perfusion in the group with 2-hour oxygenation at the start of HMP oxygenated by a membrane oxygenator compared with standard non-HMPO₂ and the bubble and intermittent surface oxygenation groups (Figure 5).

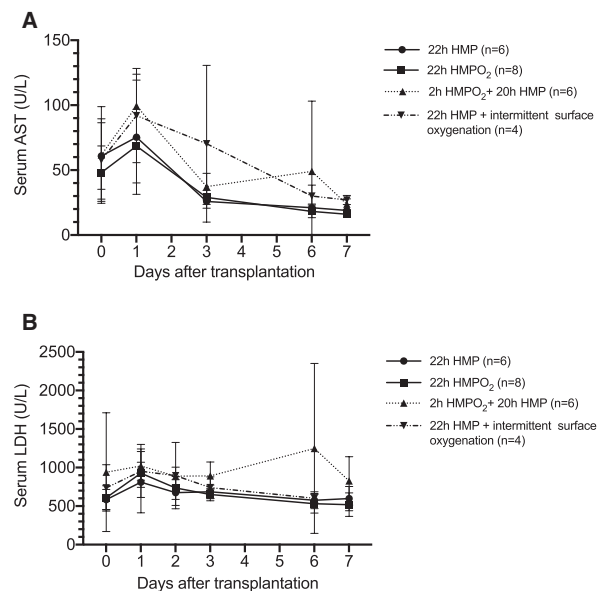


FIGURE 3. Evolution of serum AST and LDH. Evolution of serum AST (A) and LDH (B) after transplantation in all study groups. AST, aspartate aminotransaminase; HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion; LDH, lactate dehydrogenase.

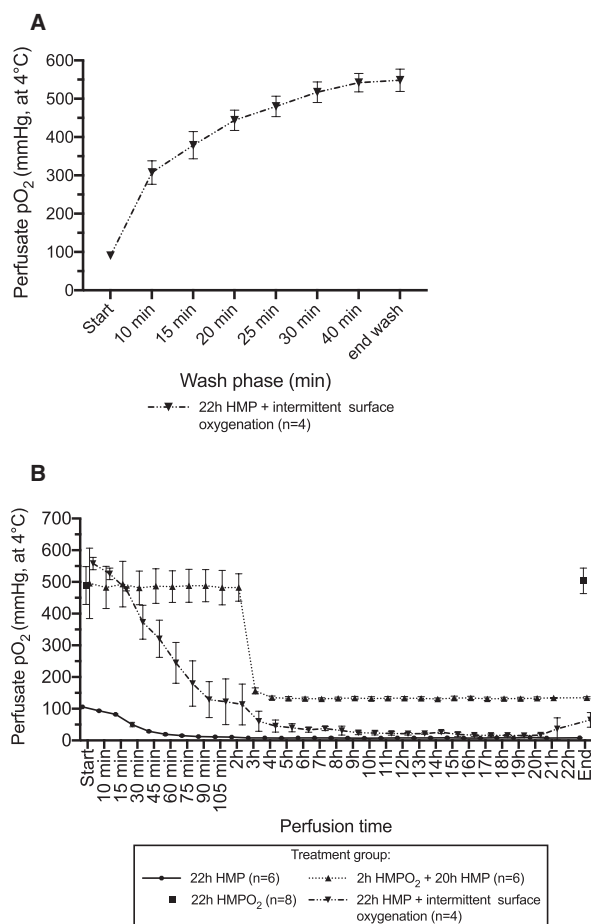


FIGURE 4. Perfusate pO₂ during machine perfusion. Direct perfusate oxygenation by bubble oxygenation during the perfusion circuit setup demonstrates pO₂ concentrations of >400 and 500 mmHg at 4°C after 20 and 30 min, respectively (A). Continuous pO₂ measurements during kidney perfusion shows that 30 min of surface oxygenation at the start of HMP was able to maintain supraphysiological pO₂ levels during the first 2 h of machine perfusion and 1 h surface oxygenation at the end of HMP demonstrates a slight increase of perfusate pO₂ (B). In contrast, perfusate pO₂ levels using a membrane oxygenator are much more static during HMP. HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion.

Metabolic Evaluation of Preservation and Reperfusion Biopsies

No differences in lactate level were observed in biopsies obtained during preservation and after reperfusion comparing both brief oxygenation groups and the standard HMP group by liquid chromatography coupled to electrospray ionization mass spectrometry analysis (Figure 6A). Succinate and glutamate levels measured at the end of the cold preservation were significantly lower when oxygen was added for 2 hours by a membrane oxygenator when compared with bubble and

intermittent surface oxygenation during HMP and standard non-HMPO₂ ($P = 0.02$ and $P = 0.04$ and $P = 0.04$ and $P = 0.01$, respectively, for succinate and glutamate) (Figure 6B and C). No differences in succinate and glutamate were observed between the HMP group without active oxygenation and the bubble and surface oxygenated group. The ATP levels, detected at the end of cold perfusion, were only significantly higher in kidneys receiving oxygen for 2 hours at start of HMP by membrane oxygenator compared with the 22-hour standard non-HMPO₂ group. Restoration of ATP

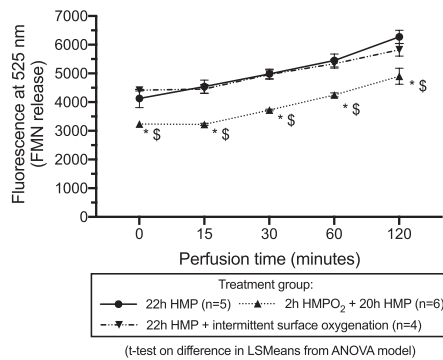


FIGURE 5. FMN detection in perfusate. FMN detection in the perfusate by fluorescence demonstrating significantly lower values during the first 2 h of machine perfusion in favor of the membrane oxygenator group compared with standard, nonoxygenated HMP or brief bubble and intermittent surface oxygenation. *Significance of $P < 0.05$ between standard 22-h HMP and 2-h HMPO₂ + 20-h HMP group. \$Significance of $P < 0.05$ between 22-h HMP + intermittent surface oxygenation and 2-h HMPO₂ + 20-h HMP. FMN, flavin mononucleotide; HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion.

levels was observed in both brief HMPO₂ groups independent of oxygenation technique. No differences in ATP levels at the end of machine perfusion were observed between both oxygenated groups ($P = 0.25$). No significant differences were observed between both HMPO₂ groups and the standard HMP group regarding ADP, AMP, and reduced nicotinamide adenine dinucleotide levels at the end of cold perfusion (Figure 6E–H). At 5 and 45 minutes after in situ reperfusion, oxidized nicotinamide adenine dinucleotide was significantly higher in kidneys treated with oxygen initially independent of O₂ administration technique when compared with standard, non-HMPO₂ (Figure 6H).

Histology: Light Microscopy

Acute tubular injury at the end of cold preservation assessed by the Hosgood score demonstrated a significant difference between all groups ($P = 0.038$) compared with no observed difference using the Banff criteria (Figure 7). No differences were observed between all groups for all other Banff criteria (eg, interstitial fibrosis, tubular atrophy, interstitial inflammation, arterial intimal fibrosis, arteriolar hyalinosis, and glomerular thrombi). No significant differences were observed between all groups in the baseline and the biopsy taken before euthanasia (data not shown).

DISCUSSION

This study, using an ischemia-reperfusion porcine kidney autotransplant model, evaluates the impact of brief bubble and intermittent surface oxygenation on early graft recovery compared with membrane oxygenation during HMP. The aim was to determine an alternative oxygenation technique (easy, cheap, and compatible with air transport) for membrane oxygenation during HMP. All oxygenated study groups, independent of oxygen administration technique and

duration of oxygenation, demonstrated superior early graft function when compared with 22-hour HMP without additional oxygenation. Brief bubble and surface oxygenation at the start of perfusion was effective to maintain supraphysiological Po₂ levels during the first 2 hours of HMP yielding improved flow dynamics. Both brief O₂ uploading oxygenation techniques demonstrated similar graft function compared with continuous membrane HMPO₂. Although the metabolic profile obtained in perfusate (ie, FMN) and kidney tissues (ie, glutamate and ATP) suggests that brief O₂ uploading for 2 hours at the start of HMP might be slightly better to protect mitochondria using a membrane oxygenator compared with bubble and intermittent surface oxygenation, both brief oxygenation techniques demonstrated similar graft function as seen in continuous HMPO₂. Bubble and intermittent surface oxygenation might be an easy alternative for membrane oxygenation eliminating the need for a membrane oxygenator and therefore reducing the total costs of oxygenated machine perfusion and making air transport easier.

A bubble oxygenator was incorporated in cardiopulmonary bypass technology during the first successful open heart surgery in 1953¹⁹ and was popular until the end 1970s because of its low cost and applied for short cardiac surgery.^{19,20} The idea of a protective membrane between blood and air to decrease the problem of blood trauma resulted in the development of membrane oxygenators, commercially available from 1960.^{19,20} From the beginning of the 1980s, hollow fiber membrane oxygenators, which allowed even more optimal gas exchange, replaced bubble oxygenators as extracorporeal oxygenators in most clinical applications.^{19,20} For this reason, a neonatal hollow fiber membrane oxygenator was used in our previous research projects to explore the effect of oxygen in different normothermic and HMP strategies.^{6,9,10} The rationale for omitting the neonatal membrane oxygenator was based on its oxygen transfer capacity being significantly higher than what is needed for sustaining aerobic metabolism of a single kidney at $\pm 4^{\circ}\text{C}$. The average O₂ transfer of 14.8 mL O₂/min of the Dideco kids D100 neonatal oxygenator²¹ far exceeds the O₂ consumption of a 100–200 g kidney in the cold which is being estimated at 0.68 mL O₂/min.²²

Today, machine preservation solutions are acellular without oxygen transporter and oxygen must reach the organ tissue by diffusion. To make organ oxygenation during HMP logistically easier, we had renewed interest in bubble and surface oxygenation to raise the dissolved perfusate O₂ concentration. This technique is based on 4 principles. First, bubble oxygenation is directly proportional to the oxygen volume and inversely proportional to the bubble size. This results in a highly effective O₂ transfer.²³ Although continuing bubble oxygenation of the perfusate during kidney perfusion would have been highly effective to maintain supraphysiological Po₂ levels, it was halted because it could cause massive foam formation due to protein degradation during kidney perfusion. Second, the solubility of oxygen increases as temperature decreases.²⁴ Third, according to Henry's law, oxygen will slowly diffuse across the surface of the perfusate from the gaseous compartment on the top of it. The amount of O₂ diffusing into the perfusate will be proportional to the percent (=partial pressure) of O₂ above its surface. And fourth, the efficiency of surface oxygenation was enhanced during the regularly scheduled wash cycles (every 10 min) during machine perfusion resulting in breaking the perfusate's surface layer. The

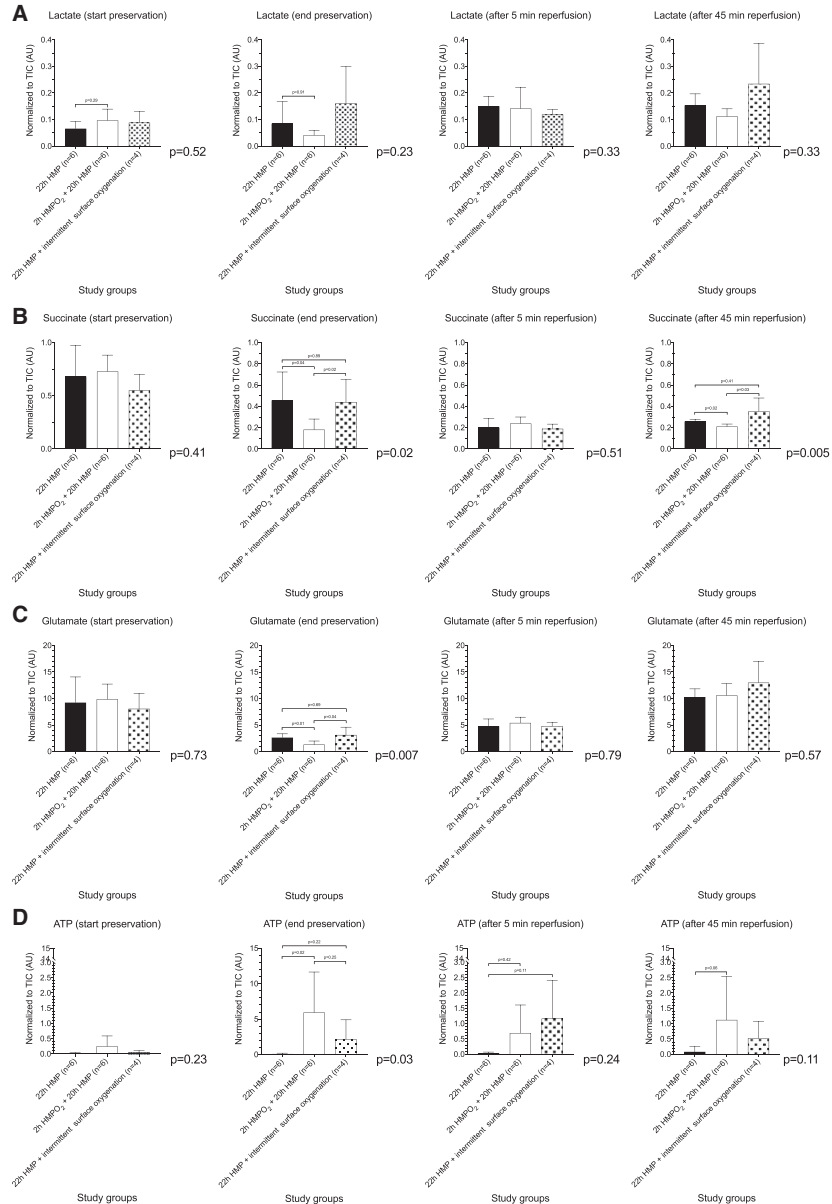


FIGURE 6. Metabolic tissue profile analysis. Metabolic profile analysis [lactate (A), succinate (B), glutamate (C), ATP (D), ADP (E), AMP (F), NADH (G), NAD⁺ (H)] on preservation and reperfusion biopsies by liquid chromatography electrospray ionization mass spectrometry demonstrates that active oxygenation during HMP realized by a membrane oxygenator seems better to support aerobic metabolism, in particular the central major metabolites succinate, glutamate, and the end product ATP when compared with bubble and intermittent surface oxygenation and standard, nonoxygenated HMP. Statistical analysis was performed using Kruskal-Wallis and Mann-Whitney tests. HMP, hypothermic machine perfusion; HMPO₂, oxygenated hypothermic machine perfusion.

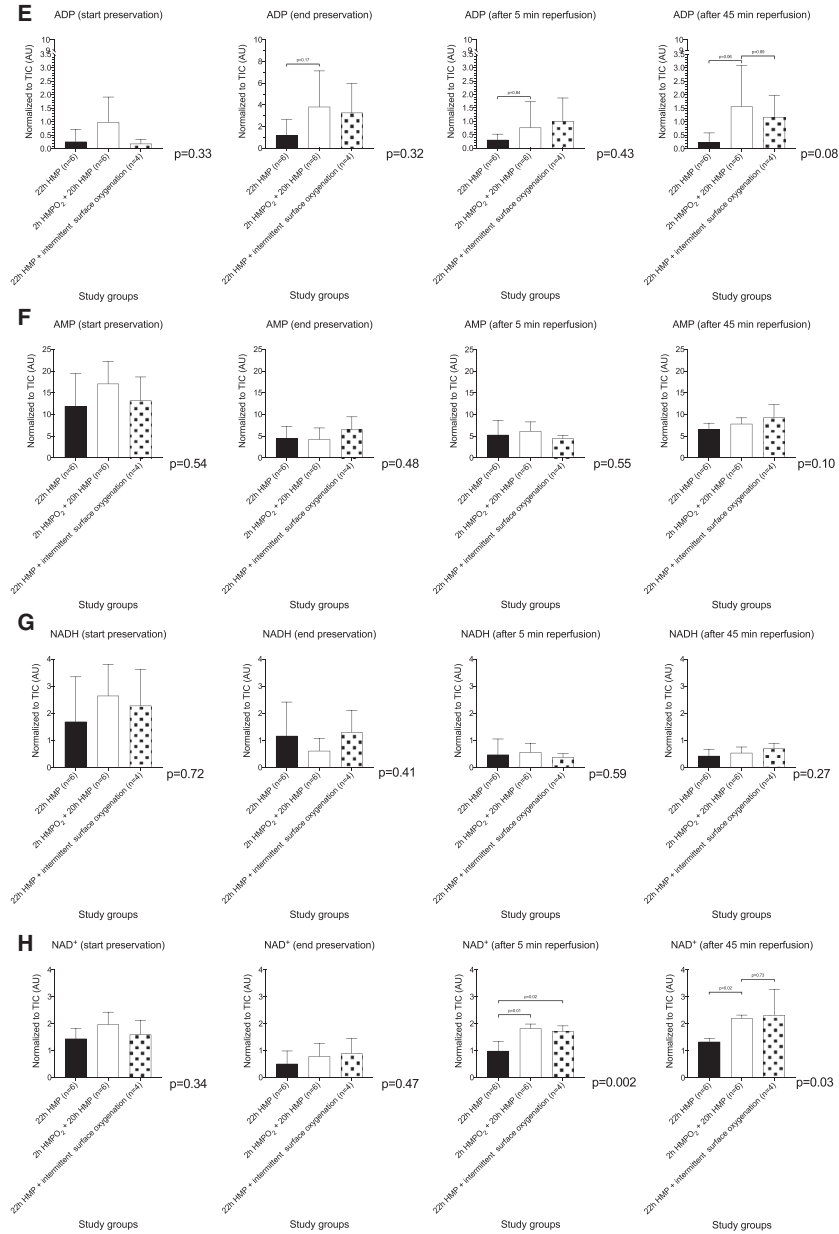


FIGURE 6. Continued.

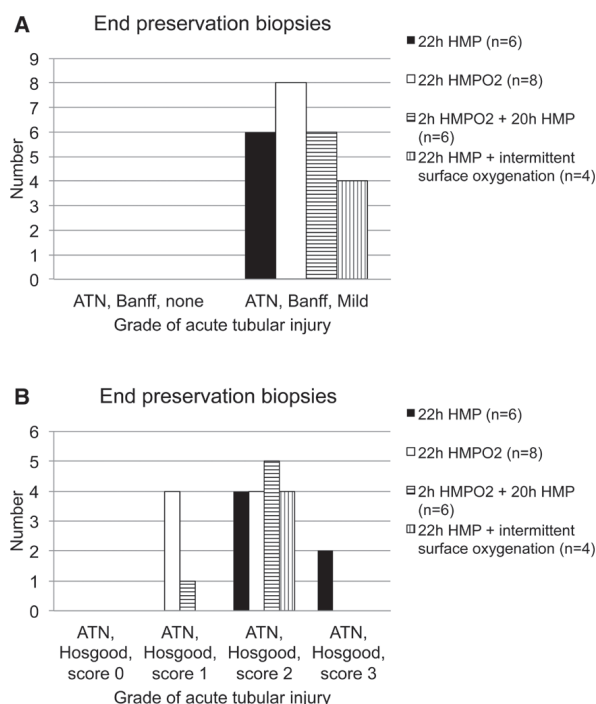


FIGURE 7. Acute tubular injury evaluation on end preservation biopsies. Acute tubular injury at the end of cold preservation assessed by the Banff criteria demonstrated no difference (A) compared with a significant difference between all groups ($P = 0.038$) using the Hosgood score (B). ATN, acute tubulus necrosis; HMP, hypothermic machine perfusion; HMPO₂, oxygenation hypothermic machine perfusion.

stream of perfusate exiting the port of the bypass line during this wash cycles causes an active mixing effect facilitating the entry of oxygen into the perfusate.

A paired international randomized controlled trial demonstrated a clinically relevant improvement in glomerular filtration rate at 12 months when considering the effect of continuous oxygenated perfusion on graft survival (mean [SE] 47.6 [1.9] versus 42.6 [2.0] mL/min/1.73 m², $P = 0.035$). The beneficial effect of oxygenated perfusion may be mediated via the reduction of organ rejection (14% versus 28%, $P = 0.014$), with a 50% reduction in acute rejection observed in the oxygenated arm. No difference was observed in early kidney function as measured by the occurrence of delayed graft function (38% in both groups).²⁵ In our study, bubble oxygenation in combination with 30-minute surface oxygenation at the start of HMP demonstrated to be highly effective to raise and maintain perfusate Po₂ concentrations above physiological level (Figure 4A–B). These observations are in line with the results of Lazeyras et al.⁷ Although we could have opted to continue surface oxygenation for 2 hours or even throughout the entire perfusion time, we decided to stop active surface oxygenation after 30 minutes of perfusion. We based this decision on previous work showing that 2 hours of active oxygenation at the start of 22-hour HMP was efficient to correct O₂ and ATP debts.¹⁰ The

reason to stop surface oxygenation after 30 minutes was also driven by clinical considerations. It is most common that after 30 minutes of perfusion the machine-perfused kidney is about to leave the donor hospital, being sent to the recipient's transplant center. By demonstrating that active oxygenation would no longer be required once oxygen and ATP debts have been fully corrected and perfusate has been fully uploaded with oxygen, one would avoid membrane oxygenation and an external oxygen source during transport while exchanging oxygenated machine-perfused kidneys between centers.

Mitochondrial succinate accumulation originating from the citric acid cycle during ischemia is one of the main contributors for superoxide generation by reverse electron transport during subsequent in vivo reperfusion under normothermic conditions.^{26–28} We previously described how oxygen during different HMPO₂ strategies resulted in more forward electron transport at the mitochondrial oxidative phosphorylation chain with superior mitochondrial protection at the end of preservation and superior subsequent in vivo early graft function compared with standard, non-HMPO₂.¹⁰ In this current study, brief oxygenation at the start of HMP demonstrated that mitochondrial protection might be slightly superior using a membrane oxygenator for 2 hours when compared with brief bubble and intermittent surface oxygenation. This might

be explained by the differences in perfusate Po_2 levels during the first 2 hours of HMP. Membrane oxygenation results in a higher and more constant perfusate Po_2 level when compared with 30 minutes of surface oxygenation demonstrating a faster drop in Po_2 levels in the first 2 hours of machine perfusion. Therefore, forward electron transport at the mitochondrial oxidative phosphorylation chain might be lower during HMP using surface oxygenation compared with membrane oxygenation. In practice, the dissolved oxygen in the perfusate can be increased by 2 strategies. First, according to Henry's law, a higher Po_2 concentration at the surface of the perfusate will increase the perfusate oxygen concentration during HMP. This could be realized by an air pump insufflating continuously room air or carbogen during organ transport. Another way to realize this in clinical practice is to interrupt surface oxygenation only for organ transport and restart surface oxygenation from the moment the machine-perfused kidney arrived in the recipient center until transplantation. Second, a different approach is to further enhance O_2 delivery to increase flow during HMP. This could be achieved by adding vasodilators in the perfusate as demonstrated during liver preservation.²⁹⁻³¹

We recognize the limitations inherent to this autotransplant model as mentioned previously.^{6,9} The use of historical control groups might have introduced a relevant bias due to slightly shorter anastomosis time in the experimental study group compared with 2 of the 3 historical groups but was recommended by the ethical committee to reduce animal numbers and suffering. Because total preservation time was comparable between all study groups, we do not expect to have an important influence on outcome measures. The difference in duration of active oxygenation in the 2 intermittent oxygenated study groups must be taken into account interpreting the metabolic profile analysis. Another limitation is that oxygen consumption during HMP was not measured.

In conclusion, the data presented in this feasibility study support the conclusion that brief bubble and intermittent surface oxygenation could be an alternative oxygenation technique to achieve improved kidney graft function compared with HMP without active oxygenation and similar functional outcome when compared with membrane HMPO_2 . This alternative oxygenation technique has the potential to decrease the total costs of HMPO_2 by eliminating the membrane oxygenator and making air transport easier. However, translation of these animal results into clinical practice must be done with caution. Further experiments need to determine the optimal duration of surface oxygenation during HMP to improve mitochondrial protection before its introduction into clinical trials.

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Summary Paper 4

Aim of the study:

The aim of this feasibility study was to determine an alternative oxygenation technique (easy, cheap and compatible with air transport) for membrane oxygenation during hypothermic machine perfusion (HMP) to improve early graft function in a porcine ischemia-reperfusion DCD autotransplant model.

Main conclusions:

- The data presented in this feasibility study support the conclusion that brief bubble and intermittent surface oxygenation could be an alternative oxygenation technique during HMP to achieve improved kidney graft function compared with HMP without active oxygenation and similar functional outcome as compared with membrane-oxygenated HMP.
- Brief bubble and surface oxygenation at the start of perfusion was effective to maintain supraphysiological oxygen concentration in the preservation fluid during the first 2 hours of HMP yielding improved flow dynamics.
- This alternative oxygenation technique has the potential to decrease the total costs of oxygenated HMP by eliminating the membrane oxygenator and need for an oxygen source during organ transport (and therefore making air transport easier).

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Kidney transplantation is the "gold standard" treatment for end-stage kidney disease, providing superior outcomes in patient survival (when compared with maintenance dialysis), quality of life and cost reduction compared to chronic dialysis.¹⁻¹¹ During the last 2 decades, in response to the organ shortage, kidney grafts from brain death extended criteria donors and from donation after circulatory death are frequently accepted for transplantation. However, these "suboptimal" organs result in a higher rate of primary nonfunction, delayed graft function (DGF) and increased rates of early graft loss.¹²⁻²⁰ The short-time clinical consequences are the need for several posttransplant dialysis sessions leading to increased morbidity, increased length of hospitalization, and hence increased costs.^{19,21-24} To improve graft preservation compared with static cold storage, which is still worldwide the "gold standard" for kidney preservation, dynamic preservation strategies have been reintroduced as a method of organ preservation with the aim to: 1) recondition/repair and evaluate transplantability of the kidney during preservation, 2) decrease the ischemia-reperfusion injury and the incidence of DGF, and 3) improve early and late graft function.

The overall aim of this thesis was to evaluate the optimal start time, temperature, oxygen concentration and oxygen administration technique during ex vivo machine perfusion in a porcine ischemia-reperfusion autotransplant model to improve early kidney graft function compared with static cold storage alone. With the help of different teams (SA Hosgood and ML Nicholson, AddenBrooke's Hospital, Department of Surgery, Cambridge, United Kingdom; Peter De Meylder, Organ Recovery System, Diegem, Belgium; Prof M Mourad, Prof. P Gianello and M Vergauwen from the Institut de Recherche Expérimentale et Clinique (IREC), Pôle de Chirurgie Expérimentale et Transplantation (CHEX), Université catholique de Louvain, Brussels, Belgium), we were able to set up a pig kidney autotransplant model to explore different hypothermic and normothermic machine perfusion strategies. The same model was used for the 4 studies described in this thesis.

In the **first study** we observed that hypothermic machine perfusion (HMP) applied from time of kidney procurement until transplantation and regardless of supplemental oxygenation (Carbogen 30% (95% O₂ / 5% CO₂)) led to a significant positive effect on early graft function compared

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with static cold storage alone and compared with different "end-ischemic" preservation strategies (including hypothermic oxygenated perfusion and normothermic perfusion).²⁵ The overall treatment effect on posttransplant serum creatinine of each preservation strategy demonstrated a significant difference in favor of the 22-hours continuously oxygenated HMP group compared with the standard HMP group without active oxygen supply during preservation. In addition, active oxygenation seems to be an important modulator for a faster increase in renal flow during machine perfusion. This effect was obtained during continuous HMP applied from procurement until transplantation but not when applied as an end-ischemic reconditioning strategy following a longer period of static cold storage.

Translation of these results into clinical practice suggests that kidney preservation and reconditioning should be started in optimal conditions immediately after ex vivo donor blood flush and subsequent procurement and maintained on HMP conditions during the whole period of preservation to provide the best initial graft function. In addition, this effect could be even more pronounced by the additional supply of a low dose of oxygen during standard HMP. These results do not mean that end-ischemic strategies should be abandoned. Normothermic perfusion remains an interesting tool to evaluate transplantability²⁶, and was already demonstrated to be effective after a period of static cold storage preservation to decrease the DGF rate in a single center study.²⁷ Results of the ongoing multicenter randomized controlled trial in the UK to evaluate normothermic perfusion as an end-ischemic reconditioning strategy after static cold storage are expected in 2020-2021. This will provide us more insights on how and when to use this preservation strategy in clinical practice.²⁸ Also end-ischemic reconditioning by standard HMP after a longer period of static cold storage should not be abandoned. Especially in transplant programs with a high incidence of DGF and/or for logistical reasons, a short period of HMP after a longer period of static cold storage might result a superior early graft function of as illustrated by Gallinat et al.²⁹ However, the implication of HMP from the start of procurement until transplantation on a national level in France in 2012, demonstrates that the transport of a machine-perfused kidney is today not any more a logistic issue, even over large geographical areas where airplane travel is needed.³⁰ This study of Savoye et al confirmed the reduction in DGF (odds ratio 0.23 (0.40-0.60)($p < 0.001$) occurrence among extended criteria donors after brain

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death preserved by HMP (n=801) compared with static cold storage (n=3515). The effect of oxygenated HMP end-ischemic after a period of static cold storage has recently been investigated in a multicenter international randomized controlled clinical trial (POMP trial, number ISRCTN63852508) initiated by the Consortium on Organ Preservation in Europe (COPE). Kidneys from extended criteria brain death donors were randomized on arrival at the recipient center to a minimum of 2-hours preimplantation oxygenated HMP versus continued static cold storage alone. One year graft survival was comparable in both groups (92.1% (n=117) in the end HMPO₂ group versus 93.3% (n=126) in the SCS group (p=0.7)). The secondary endpoint analysis showed similar results in both groups for delayed graft function, primary nonfunction, patient survival and episodes of acute rejection (Peri Husen, abstract presented at the British Transplant Society meeting 2020 and at the American Transplant Congress 2020).

Historically, mitochondrial reactive oxygen species production during ischemia-reperfusion injury has been considered to be a nonspecific response to reperfusion. Recent metabolomic analysis by Chouchani suggesting that selective accumulation of the citric acid cycle intermediate succinate is a universal metabolic signature of ischemia in a range of tissues and is responsible for mitochondrial reactive oxygen species production during reperfusion.^{31,32} The presence of oxygen could potentially increase the production of reactive oxygen species and subsequent ischemia-reperfusion injury.³³ Although several preclinical studies³⁴⁻³⁸ have similarly shown a beneficial effect of supplemental oxygenation (continuous or end-ischemic) for early graft function, there is evidence signifying the promotion of aerobic metabolism and restoration of cellular ATP only occurs at higher oxygen concentrations.^{39,40} These research topics were explored more in detail in the next 3 studies of the thesis.

In the **second study** we evaluated the impact of different supraphysiological perfusate "oxygen concentrations" during continuous HMP, applied from procurement until transplantation, on early graft function. Carbogen (95% O₂ / 5% CO₂) was used for oxygen supplementation at either 30% or 90% in the low- and high oxygenated group, respectively and compared to standard HMP without active oxygen supply. High pO₂ resulted in a 3- and 10-fold increase in perfusate pO₂ compared with low HMPO₂ and standard, HMP, respectively. Both HMPO₂

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groups were associated with superior initial graft recovery compared with standard HMP and static cold storage.⁴¹ Active oxygenation, independent of oxygen concentration was associated with a more rapid and sustained increase in ex vivo renal flow during machine perfusion. Whilst there was no difference in functional outcomes between both HMPO₂ groups, there were clear metabolic differences with an inverse correlation between oxygen provision and the concentration of central metabolites (lactate, succinate, formate, acetate) in the perfusion fluid but no differences were observed by oxidative stress (Nicotinamide Adenine Phosphate Oxidase 4 (NOX4), Heme Oxygenase 1 (HO-1), Superoxide Dismutase 1 (SOD1) and peroxiredoxin 1 (Prdx1)) and metabolic evaluation (lactate, ATP, ADP, AMP and reduced nicotinamide adenine dinucleotide (NADH) and oxidized nicotinamide adenine dinucleotide (NAD⁺)) on preimplantation biopsies. These results confirm previous preclinical studies^{35,40,42} demonstrating that aerobic mechanism are better supported under high oxygen concentrations during cold perfusion. Dutkowski et al recently described that end-ischemic oxygenated hypothermic liver perfusion results in forward electron transport at the level of the mitochondrial respiratory chain during HMP and leads to better mitochondrial preservation during cold perfusion.⁴³ Our study results are in line with these observations. In addition, Hypoxia-inducible factors-1 α (HIFs-1 α) are the universal cellular oxygen-sensitive transcription factors that activate a number of hypoxia-responsive genes, including vascular endothelial growth factor, erythropoietin, epidermal growth factor receptor and platelet-derived growth factor. HIF-1 α normally accumulates during ischemia but was significantly decreased in our study in the presence of active oxygenation during HMP. Therefore, HIF-1 α expression could play a major role in early and late allograft function after oxygenated HMP, as also previously described.⁴⁴

The effect of continuous oxygenated HMP was recently investigated in a multicenter, international randomized controlled trial (COMPARE trial, registration number, ISRCTN32967929) initiated by the Consortium of Organ Preservation in Europe (COPE) using the Kidney Assist (Organ Assist, Groningen, The Netherlands). One hundred and 6 paired kidneys originated from DCD donors who were 50 years or older demonstrated a clinically relevant improvement in glomerular filtration rate at 12 months when considering the effect of oxygenated perfusion on graft survival (improved 1-year creatinine clearance by 7.1 ml/min (p=0.02). The

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beneficial effect of oxygenated perfusion may be mediated via the reduction of organ rejection (14% versus 28%, $p=0.01$), with a 50% reduction in acute rejection observed in the oxygenated arm. No difference was observed in early kidney function as measured by the occurrence of DGF (38% in both groups).⁴⁵

It remains unclear if additional oxygen is required during the entire preservation period to protect mitochondria during HMP. Therefore, in the **third study**, we evaluated the impact of start time and duration of active oxygenation during continuous HMP, applied from moment of procurement until transplantation. The aim was to determine the optimal conditions for hypothermic kidney preservation to provide first an effective oxygenation and secondly an easy approach to simplify logistics during organ donation and transport. Two-hour perfusate oxygenation, right at the start of HMP led to significantly improved early renal function when compared with 22-hours standard HMP without additional oxygen supply. Interestingly, perfusate oxygenation at the start of HMP achieved comparable outcomes as seen with continuous oxygenation during HMP. This effects was not observed when adding oxygen during the last 2 hours of HMP. The established metabolic profile obtained in perfusate (i.e. lactate, succinate and flavin mononucleotide) and kidney tissues (i.e. succinate, glutamate, ATP, Nuclear factor erythroid 2-related factor 2 (Nrf2) and HIF-1 α) suggests that brief O₂ uploading at the start of HMP could be an easy and more beneficial preservation strategy to protect mitochondria and improve early graft function as compared with standard HMP without additional oxygenation and O₂ supply only at the end of the HMP period. These results supports the evidence of Chouchani et al that mitochondrial succinate accumulation during ischemia is one main contributor for superoxide generation by reversed electron transport during subsequent in vivo reperfusion under normothermic conditions.^{31,32} Dutkowski et al described this mechanism assessed during different human liver perfusion strategies.⁴³ In addition, our study results provides corroborative metabolic evidence in both perfusate and tissue analysis that similar pathological processes occur during cold kidney perfusion which can be improved via oxygenation (as illutrated in figure 9 of chapter 3, results section, personal contributions). Recent literature even suggests that it is not the accumulated ischemic mitochondrial succinate but more likely, the reduced or semi-reduced flavin located at the nucleotide-binding site, that appears

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as the direct source of reactive oxygen species production at mitochondrial complex I.⁴⁶ In 2019, flavin mononucleotide (FMN) is described for the first time by Muller et al to predict liver function already during hypothermic oxygenated liver perfusion before implantation.⁴⁷ Flavin mononucleotide determination during HMP by simple fluorescence is far more easy and faster than any currently available succinate measurement and could therefore potentially be used as real time surrogate marker to assess the metabolic status and predict ischemia-reperfusion injury and initial graft function. Our study results are the first in literature that confirms the possible value of FMN determination during hypothermic kidney machine perfusion as an interesting biomarker to predict ischemia-reperfusion injury and subsequent early graft function. The main advantage of this technique is that real time fluorescence is relatively easy to incorporate in the existing commercially available hypothermic machine perfusion devices. We do realize that the perfusate FMN measurement by fluorescence only detect released FMN dislocated from mitochondrial complex I and does not detect the FMN which is still bounded in complex I. Because the proportion of released FMN originated from ischemic cell lyses is probably related to the duration of machine perfusion, we focused on FMN measurement during the 2 first hours of HMP, which correlated well with initial graft function in our study. The value of perfusate FMN as biomarker during HMP to evaluate transplantability or early graft function needs to be further explored in clinical kidney transplant programs.

Practically, both, brief initial and end-ischemic oxygenation might be easy to implement in clinical practice. Kidneys originated from donors after circulatory death with more ATP debt at the start of preservation, compared with kidneys originated from brain death donors, probably benefit more from brief initial O₂ uploading at the donor hospital. Although, in our study, initial oxygen supply was not superior compared to continuous oxygenation, such short cold perfusion with oxygen could be of interest because it eliminates the need for an oxygen source for HMP during kidney transport. Concerning end-ischemic oxygenation during HMP, it is unknown if the beneficial effect of oxygen maintains using shorter preservation periods as in most clinical programs compared with the model used in this study.

Finally, in the **fourth study**, the identification of an efficient alternative oxygenation technique for membrane oxygenation represents a substantial

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contribution to improve early graft function in our model. Indeed, we evaluated the use of an new alternative administration technique for oxygen, namely direct bubble and surface oxygenation of the perfusion fluid at the start of continuous HMP as alternative method for brief O_2 by a classical hollow fiber membrane oxygenator (as used in the first 3 studies of this thesis). All oxygenated study groups, independent of oxygen administration technique and duration of oxygenation, demonstrated superior early graft function when compared with 22-hours standard HMP without additional oxygen supply. Brief bubble and 30 minutes surface oxygenation at the start of machine perfusion was effective to maintain supraphysiological pO_2 levels during the first 2 hours of HMP with improved flow dynamics. Both brief O_2 uploading oxygenation techniques demonstrated similar graft function compared with continuous membrane oxygenated HMP. Although, the metabolic profile obtained in perfusate (i.e. FMN) and kidney tissues (i.e. glutamate and ATP) suggests that brief O_2 uploading at the start of HMP might be slightly better to protect mitochondria using a membrane oxygenator compared with bubble and surface oxygenation. Besides the equal primary outcome measure, bubble and intermittent surface oxygenation of the perfusate during HMP turned out to be an easy and efficient alternative oxygenation method compared with membrane oxygenated-perfused kidneys. This alternative oxygenation technique has the potential to decrease the total costs of oxygenated HMP by eliminating the membrane oxygenator and oxygen source during organ transport and making (air) transport easier. However, transplation of these animal results into clinical practice must be done with caution. Further experiments need to determine the optimal duration duration of surface oxygenation during HMP to improve mitochondrial protection before its introduction into clinical trials.

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IV. Future perspectives

The effect of oxygen in different kidney preservation strategies to decrease ischemia-reperfusion injury and to improve early kidney function after transplantation was the main study objective throughout this thesis.

Normothermic machine perfusion

With the increase in temperature, the complexity of the preservation technique also increases. End-ischemic normothermic machine perfusion after a period of static cold storage or even after HMP is technically demanding and increases significantly the total cost of organ preservation. However, this technique stays an interesting tool to assess transplantability of a marginal or previous discarded organ to increase the donor pool and enhance early graft function in such kidneys.¹ In addition, normothermic perfusion offers an excellent tool for organ repair (e.g. pharmacodynamic interventions, cell therapy, molecular or genetic therapy) to decrease the harmful effects of ischemia-reperfusion injury. As mentioned before, the ongoing multicenter randomized clinical trial in the United Kingdom will provide future directives to implement this preservation strategy on a larger scale.² Another preservation strategy is to start normothermic machine preservation immediately after in situ organ cooling by a cold perfusion fluid and subsequent procurement of the organ in the donor and to continue normothermic perfusion until transplantation to minimize the harmful effects of cooling. This strategy is recently explored in a multicenter randomized clinical trial in liver transplantation demonstrating a 50% lower level of graft injury (measured by hepatocellular enzyme release), despite a 50% lower rate of organ discard and a 54% longer main preservation time in favor of the normothermic machine-perfused liver grafts (n=170) compared with the static cold storage preserved liver grafts (n=164).³ An even more optimal strategy is to avoid ischemia-reperfusion injury by not stopping the blood supply before organ procurement in the donor. This technique is called "ischemia-free organ transplantation" and was published in 2018 for the first time by He X et al.⁴ A liver graft with severe macrovesicular steatosis from a 25-year old man was procured, preserved and implanted under continuous normothermic machine perfusion in a 51-year old man with decompensated liver cirrhosis and hepatocellular carcinoma. The recipient did not suffer ischemia-reperfusion syndrome or vasoplegia after revascularisation of the allograft. No rejection, or vascular

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or biliary complications occurred. The same author published in 2019 the first case of a successfully ischemia-free kidney transplantation.⁵ A kidney graft of a 19-year old brain-dead donor was transplanted to a 47-year-old man with end-stage diabetic nephropathy without cessation of blood supply using normothermic machine perfusion. The creatinine dropped rapidly to normal range within 3 days post-transplantation. No rejection or vascular or infectious complications occurred. This strategy looks promising, however, some major limitations have to be resolved to introduce this preservation strategy on a larger scale in clinical kidney transplant programs. Firstly, a portable normothermic machine perfusion device for kidneys for this procedure is not yet commercially available. This strategy is therefore at this moment only possible when donor and recipient are co-located on a 'self made' normothermic perfusion set up. Secondly, it's difficult to conduct this technique for both kidneys from the same donor.

Oxygenated hypothermic machine perfusion

As mentioned in the discussion sections of the 4 studies in this thesis, our results are in line with recent preclinical and clinical evidence in the field of liver⁶⁻⁸ and kidney preservation demonstrating that active oxygenation during HMP provides superior mitochondrial protection with subsequent less ischemia-reperfusion and improved initial graft function as compared with standard HMP without active oxygenation and static cold storage preservation. The COPE-COMPARE trial (see general discussion and conclusions, personal contributions) in the first randomized clinical trial in kidney transplantation demonstrating a superiority of oxygenated HMP compared to standard HMP without active oxygen supply when performed during the whole cold preservation time. Based on these results, many transplant centers will be convinced to add oxygen during cold machine preservation.

Brief O₂ uploading at the start of standard HMP is a alternative oxygenation strategy in organ transplantation for membrane oxygenation and not yet explored by other research groups. Based on our preclinical results, this approach is worth to be further explored in preclinical and clinical practice, independent of the oxygen administration technique. The fourth study of this thesis was part of an international collaborative research project of Organ Recovery Systems (Diegem, Belgium). Multiple preclinical studies to explore bubble and surface oxygenation, as alternative oxygen

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administration technique for membrane oxygenation during HMP, were performed in scientific collaboration with the Birmingham, Madrid and Paris groups to reduce animal use and optimize the expertise of each research center. Based on these study results, the perfusion kit of the currently clinically used LifePort Kidney Transporter is optimized to make perfusate oxygenation possible by bubble and surface oxygenation (Figure S1, article 4). This new kidney perfusion kit is actually available to be tested in clinical transplant programs in Europe in 2020-2021 (CE approved from 9-2020). Based on our preclinical results we believe that brief O₂ uploading at start of HMP is absolutely necessary to correct ATP debt at the start of cold perfusion and to keep succinate and flavin mononucleotide (FMN)(both responsible for reactive oxygen species production after in vivo reperfusion of the organ) levels low from the start of machine perfusion to have a major impact on mitochondrial preservation and subsequent ischemia-reperfusion injury. As demonstrated in the fourth study of this thesis, surface oxygenation can probably safely be stopped during organ transport once initial ATP debt is corrected and the kidney is completely cooled between 3 and 8 °C. Because organ transport is most often much shorter in clinical practice compared with the 22-hours total preservation time in our autotransplant model, we believe that to optimise this oxygenation method, surface oxygenation should be restarted once the machine-perfused kidney arrives in the recipient center. Longer active surface oxygenation results in a superior mitochondrial protection due to higher ATP production and subsequent lower mitochondrial succinate and FMN release at the end of cold machine preservation. The optimal duration of surface oxygenation during HMP to improve mitochondrial protection and subsequent early graft function will be explored in future preclinical and clinical studies.

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V. Summary

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The overall aim of this project was to evaluate the optimal start time, temperature, oxygen concentration and oxygen administration technique during machine perfusion compared with static cold storage preservation in a porcine ischemia-reperfusion DCD autotransplant model to decrease ischemia-reperfusion injury and subsequently to improve early kidney graft function.

In the **first study** we observed that hypothermic machine perfusion (HMP) applied from time of kidney procurement until transplantation and regardless of supplemental oxygenation (30% carbogen) led to a significant positive effect on early graft function compared with static cold storage alone and compared with different end-ischemic preservation strategies. The overall treatment effect on posttransplant serum creatinine of each preservation strategy demonstrated a significant difference in favor of the continuously oxygenated HMP group compared with the standard HMP group without active oxygen supply during preservation. In addition, active oxygenation was found to be an important modulator for a faster increase in renal flow during machine perfusion.

Investigating in the **second study** the impact of different oxygen concentrations during continuous HMP applied from procurement until transplantation on early graft function, we observed that high pO_2 (90% carbogen) resulted in a 3- and 10-fold increase in perfusion fluid pO_2 compared with low $HMPO_2$ (30% carbogen) and standard HMP without active oxygenation, respectively. Both $HMPO_2$ groups were associated with superior initial graft recovery compared with standard HMP and static cold storage. Active oxygenation, independent of oxygen concentration was associated with a more rapid and sustained increase in ex vivo renal flow during machine perfusion. Whilst there was no difference in functional outcomes between both $HMPO_2$ groups, there were clear metabolic differences with an inverse correlation between oxygen provision and the concentration of central metabolites (lactate, succinate, formate, acetate) in the perfusion fluid but no differences were observed by oxidative stress (Nicotinamide Adenine Phosphate Oxidase 4 (NOX4), Heme Oxygenase 1 (HO-1), Superoxide Dismutase 1 (SOD1) and peroxiredoxin 1 (Prdx1)) and metabolic evaluation (lactate, ATP, ADP, AMP and reduced nicotinamide adenine dinucleotide (NADH) and oxidized nicotinamide adenine dinucleotide (NAD^+)) on preimplantation biopsies.

Interestingly, in the **third study**, brief perfusion fluid oxygenation at the start of HMP achieved comparable outcomes as seen with continuous oxygenation during HMP. This effect was not observed when adding

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oxygen during the last 2 hours of HMP. The established metabolic profile obtained in the perfusion fluid (i.e. lactate, succinate and flavin mononucleotide) and kidney tissues (i.e. succinate, glutamate, ATP, nuclear factor erythroid 2-related factor 2 and hypoxia inducible factor-1 α) suggests that a brief O₂ uploading at the start of HMP could be an easy and more beneficial preservation strategy to protect mitochondria and improve early graft function as compared with standard HMP without additional oxygenation and O₂ supply only at the end of the HMP period. In addition, flavin mononucleotide measurement by simple fluorescence during hypothermic kidney machine perfusion demonstrated to be an interesting real time biomarker to predict ischemia-reperfusion injury and subsequent early graft function.

Finally, in a **fourth study**, our work leads us to observe that brief bubble and 30 minutes surface oxygenation of the perfusion fluid at the start of machine perfusion was effective to maintain supraphysiological pO₂ levels during the first 2 hours of HMP with improved flow dynamics and demonstrated similar graft function as compared with continuous membrane-oxygenated HMP. However, the metabolic profile obtained in the perfusion fluid (i.e. flavine mononucleotide) and kidney tissues (i.e. glutamate and ATP) suggests that brief O₂ uploading at the start of HMP might be slightly better to protect mitochondria using a membrane oxygenator compared with bubble and intermittent surface oxygenation. Besides the equal primary outcome measure, bubble and surface oxygenation of the perfusate during HMP turned out to be a user-friendly and efficient alternative oxygenation method. The implementation in clinical practice of this new oxygen administration technique might be quite easy because it hardly complicate handling and logistics as compared with standard HMP without active oxygen supply. In addition, it will also reduce the total cost of machine perfusion compared with perfusion devices using a membrane oxygenator as well as an oxygen source during the organ transport.

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* included DBD/ECD/DCD

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* included DBD/ECD/DCD

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* included DBD/ECD/DCD

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Figure 8: Overview pathophysiology ischemia-reperfusion injury. The blood flow is interrupted and causes an imbalance between O₂ delivery and demand. Hypoxia, glucose depletion, acidosis and ROS production contribute to cell death. Tubular epithelial and vascular endothelial cells release pro-inflammatory cytokines (interstitial inflammation) that are responsible for endothelial cell swelling with a consequent prolonged ischemia and slower reperfusion (interstitial microvasculopathy). During reperfusion, the restoration of pH induce Ca²⁺ delivery by depleted cells, producing Ca²⁺ overload and increased oxygen-derived free radical formation that both contribute to an inflammatory cascade and cell injury (figure from⁷¹). ATP, adenosine triphosphate; Ca⁺⁺, calcium ions, DNA, deoxyribonucleic acid; GLC, glucose, ROS, reactive oxygen species; IRI, ischemia-reperfusion injury; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.

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Figure 9: Factors influencing the degree of ischemia reperfusion injury after transplantation and subsequent graft function (figure adapted from Pirenne J, presented at the 17th Congress of the European Society for Organ Transplantation 2015). HMP, hypothermic machine perfusion, NP, normothermic perfusion; SCS, static cold storage; Tx, Transplant.

Figure 10: Currently available hypothermic machine perfusion devices for clinical kidney preservation: a) RM3® Kidney Perfusion System (Waters Medical systems, Birmingham, AL, USA), b) WAVES® transportable Kidney Perfusion (Waters Medical systems, Birmingham, AL, USA), c) LifePort Kidney Transporter® (Organ Recovery Systems, Itasca, IL, USA), d) Kidney Assist® (Organ Assist, Groningen, the Netherlands).

Figure 11: Schematic overview of the ex vivo normothermic perfusion system (a) and an clinical image of an ex vivo normothermic perfused kidney (b)(courtesy of Hosgood SA). The renal artery and vein and ureter were cannulated and the kidney was perfused with 1 unit of compatible packed red blood cells mixed with a priming solution. The kidney was perfused at a mean arterial pressure of 60 mmHg and temperature of 35°C for 60 min.

Curriculum Vitae

Curriculum Vitae

Tom Darius was born in Ranchi (India), September 29th 1977. He attended high school at the Sint Pieterscollege in Leuven, Belgium. He went on to take his degree in medicine at the KU Leuven and graduated cum laude in 2002. He then started a specialist training in general surgery at the Leuven University Hospitals under supervision of Prof. P. Broos. He obtained his degree as a general surgeon in 2008. He started a fellowship in abdominal transplant surgery under guidance of Prof. J. Pirenne from 2008 until 2010. In 2010 he joined the surgery and abdominal transplantation Unit at the University Clinic Saint Luc, Brussels as a staff member. Tom is married to Katrien Jansen and they have two children, Sofie and Anna.

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